CHARACTERIZATION OF AND RISK FACTORS FOR HIV-1 RESISTANCE MUTATIONS FOLLOWING FIRST LINE ANTIRETROVIRAL FAILURE IN THE SOUTHERN AFRICAN PRIVATE SECTOR

by

EDGAR LEONARD LUTAAYA
LTYLEO001

SUBMITTED TO THE UNIVERSITY OF CAPE TOWN

In partial fulfilment of the requirements for the degree of

Master of Medicine (MMed) in Medicine

Faculty of Health Sciences

University of Cape Town

Date of submission: 13/03/2017

Supervisors: Professor Graeme Meintjes and Dr. Carole Wallis

Department of Medicine

University of Cape Town

DECLARATION

I, ...***Leonard Edgar Lutaaya***..., hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.
I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Signature: …… ……

Date: ……**13/03/2017**…….

Contents: Page

Contents: Page

Acknowledgements

I would like to thank Professor Meintjes for he's support and patience. You've provided me with an excellent learning opportunity which I have thoroughly enjoyed.

Thanks to Dr. Carole Wallis for her valuable input and for allowing me access to the Lancet laboratories where I gained further insight into the genotyping process.

Thanks to Kathryn Manning and the team at AfA including Michael Hislop and Liezl Dunn, your hard work was much appreciated.

Thanks to my Mom and Dad for your unwavering love and support and for giving me the opportunity to educate myself.

To my wife Roslyn and my 3 children for your unconditional love and support.

To my late grandmother, Tereza Namirimu Zasanze to whom I dedicate this work, from Bunga Uganda who endured repeated loss when the scourge of HIV was at its worst. It is an honor to have contributed towards work that affected you so dearly.

Figures and Tables

Figures

Literature Review:

Manuscript:

Tables

Supplementary table 3: Unadjusted risk ratios for variables associated with NNRTI mutations regression

List of Abbreviations

AfA……...Aids for Africa

ART……..Ante-retroviral therapy

DDI……...Didanosine

D4T……...Stavudine

EFV……...Efavirenz

FTC……...Emtricitabine

HIV……...Human Immunodeficiency Virus

NRTI……Nucleoside reverse transcriptase inhibitor

NNRTI……Non-nucleoside reverse transcriptase inhibitor

NVP……...Nevirapine

SA…….....South Africa

TAM……..Thymidine analogue mutation

TDF……....Tenofovir

3TC…….....Lamivudine

Abstract

Background: First-line antiretroviral therapy (ART) regimen choices have evolved over the past 15 years in South Africa. Many patients develop HIV drug resistance mutations when they fail first-line ART. The effect of different drug regimens and other patient related factors on drug resistant mutations selected at first line failure are not well characterised in Southern Africa with its predominantly subtype C epidemic.

Objectives: To characterize HIV resistance mutations in patients failing first-line ART in the private sector in Southern Africa, and risk factors associated with resistance and particular resistance mutations.

Methods: This was a retrospective observational study linking two databases. One database was that of the Aid for AIDS (AfA) disease management programme (for clinical and ART regimen data) and the other was that of Lancet Laboratories where HIV genotypic sequence data were stored. Variables included in analyses were age, gender, province, WHO stage at time of resistance testing (HIVDR), duration of ART at HIVDR, prior monotherapy or dual therapy, viral load and CD4 count prior to starting ART, viral load and CD4 count at HIVDR, duration that the viral load was >400 copies/ml prior to HIVDR and the HIV subtype. Data on patients who had a resistance test between 2008 and 2014 while failing first-line ART was extracted from these databases. Fisher's exact test and Chi-squared test were used to analyse categorical variables and Wilcoxon rank sum test for continuous variables. For multivariate analyses, logistic regression models were used.

Results: Patients who registered with AfA between 1998 and 2013 and had a resistance test performed whilst experiencing viral failure on a non-nucleoside reverse transcriptase inhibitor (NNRTI) regimen with no history of prior failure on a protease inhibitor (PI) regimen were included. 265 (95.3%) patients had a reverse transcriptase mutation of any kind, 253 (91%) had an NNRTI mutation and 246 (88.5%) had a nucleoside reverse transcriptase inhibitor (NRTI) mutation (n=278). The commonest mutation was the M184V mutation (80.6%), 44.2% had at least one thymidine analogue mutation (TAM) and 42.1% had the K103N/S mutation. Notably there was a median of 18 months during which the viral load was not suppressed prior to the resistance tests, which likely contributed to the high prevalence of mutations. A total of 83 (29.9%) patients were found to have the K65R mutation, 72 of these patients were on tenofovir (TDF) at the time of resistance testing demonstrating the strong association between TDF and the K65R mutation.

Conclusions: The mutational patterns observed in our study and their prevalence were similar to those noted in previous studies done within the region's public sector. There was a high prevalence of the K65R mutation in patients failing TDF-containing regimens. A longer duration on ART and failing ART were correlated with an increased number of TAMs.

Literature review

Antiretroviral therapy resistance and studies of first-line resistance reported from sub-Saharan Africa

The Human Immunodeficiency Virus (HIV) epidemic and antiretroviral therapy (ART)

The spread of HIV has resulted in over 36.9 million people living with HIV worldwide by the end of 2014, 93% of whom were adults(1,2). By June 2015 a total of 15.8 million people infected with HIV were accessing anti-retroviral therapy (ART)(2). The greatest burden of disease is carried by sub-Saharan Africa where 71% of individuals with HIV reside (1). Globally, there has been a 35% decrease in new HIV infections since 2000, a 42% decrease in AIDS-related deaths since the peak in 2004 and an 84% increase in the access to ART since 2010(2). South Africa (SA) is by far the country with the most individuals living with HIV with over 6.8 million individuals estimated to be infected(3). Since 2000, there has been a rapid scale-up of ART in sub-Saharan Africa. An estimated 3 million individuals are now receiving ART in South Africa(3), which is the highest number globally and puts the country at the greatest risk for the development of resistance to ART. The ART regimens provided have undergone several changes as the knowledge of individual drug characteristics and side effects has evolved. In resource-limited settings ART options to date have been limited, however, with the development of cheaper ART drugs, over time more options are becoming available. As a consequence of ART scale-up, this has predictably been accompanied by increasing drug resistance in individuals infected with HIV. Understanding the frequency and patterns of drug resistance will inform treatment and programmatic decisions regarding drug options. This review provides a brief introduction to relevant HIV virology, ART drugs, drug resistance mechanisms and a literature review of studies that reported ART resistance in patients who failed first-line ART in sub-Saharan Africa. Figure 1 represents the worldwide distribution of the various HIV subtypes and displays the overwhelming predominance of subtype C in sub-Saharan Africa.

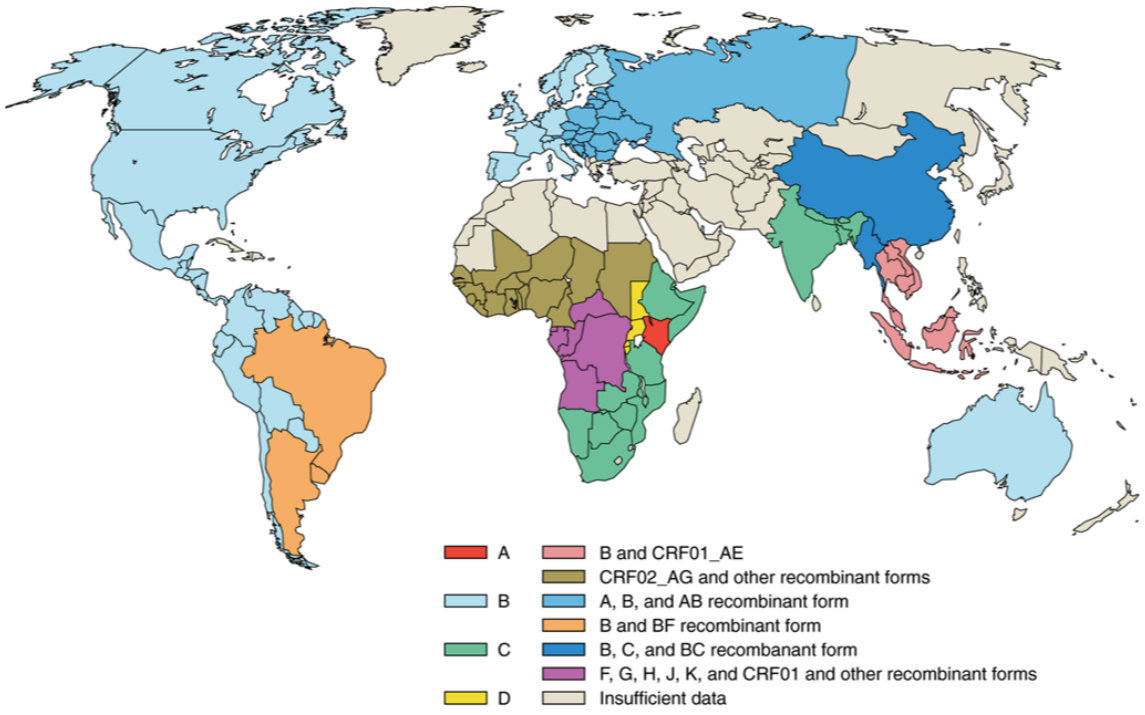


Figure 1: Global Distribution of HIV-1 subtypes (4)

HIV: virology

HIV is a human retrovirus that belongs to the lentivirus family(5). There are two genetically distinct types of HIV, namely, HIV type 1 (HIV–1) and HIV type 2 (HIV–2). HIV–1 is the predominant type and is found throughout the world(4). HIV-2 is less virulent and mainly seen in West Africa. HIV-1 can be further sub classified into M, N, O and P(4). The M subgroup is responsible for most infections worldwide and is divided into 9 clades (A, B, C, D, F, G, H, J and K(4). Subtype C is the most prevalent in sub-Saharan Africa and South Africa. HIV-2 is sub classified into groups A through to G(4).

The HIV-1 virus is surrounded by a lipid envelope that is derived from the host cell membrane(5). Within the core of the virus are several key proteins, genes and enzymes(5). These include p24 which is described as the major capsid protein, the nucleocapsid proteins p7/p9, two copies of genomic RNA and three viral enzymes namely; protease, reverse transcriptase and integrase(5).

The HIV–1 RNA genome contains several genes including the *pol*, *gag* and *env* genes(5). The *pol* and *gag* genes are translated into large precursor proteins that are cleaved into more mature proteins by the protease enzyme which is targeted by protease inhibitors(5).

HIV infects CD4 T-cells by the binding of its surface protein gp-120 to the CD4 receptor and co-receptors CCR5 or CXCR4(4,5). This attachment results in a conformation change and uncoating of the virion with penetration of the gp-41 transmembrane protein through the plasma membrane of the CD4 T cell(4,5). This results in subsequent entry of the virion into the cellular cytoplasm of the CD4 T cell(4,5). Its single-stranded viral RNA is then reverse transcribed, by the viral reverse transcriptase enzyme into complementary DNA (cDNA)(5). Thereafter double stranded DNA (dsDNA) is generated and this viral dsDNA is transferred into the nucleus of the cell by the viral integrase protein(5). The viral integrase also cleavages the host DNA and allows for the viral cDNA to be integrated into the host DNA(5). Once integrated into the host DNA the viral DNA is replicated by the host, thus, allowing for the viral proteins to be produced(5). The viral proteins are assembled in the cytoplasm and packaged into immature virions(5). These immature virions bud out of the cell and mature through cleaving of viral polyproteins by the HIV protease enzyme(5).

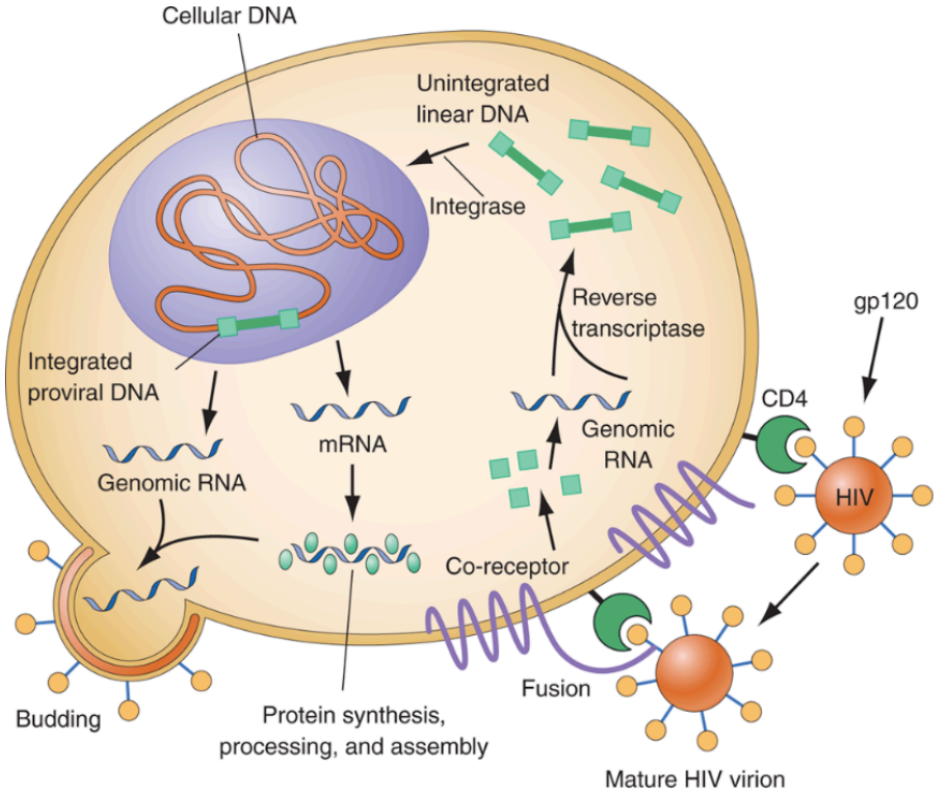


Figure 2: The HIV lifecycle (4)

ART drugs: adherence and resistance

Antiretrovirals have been developed to target the viral proteins, namely, reverse transcriptase, integrase and protease. NRTIs (nucleoside reverse transcriptase inhibitors), NNRTIs (non-nucleoside reverse transcriptase inhibitors) are the main first-line ART drugs used in SA and inhibit the reverse transcriptase (RT) enzyme. Protease inhibitors (PI's) inhibit protease enzyme that is essential for formation of infectious virions and integrase inhibitors inhibit viral DNA integration into the host cell chromosome. Newer and investigational drug classes have other targets (e.g. entry inhibitors, capsid inhibitors).

NRTIs resemble natural nucleotide building blocks of DNA and are therefore added to the developing strand of cDNA thereby inhibiting reverse transcription of the viral RNA into cDNA. NNRTIs bind to the hydrophobic pocket on the reverse transcriptase enzyme thereby inhibiting the viral RNA from binding to the RT enzyme.

In sub-Saharan Africa first-line ART typically comprises of two NRTIs and 1 NNRTI. The most commonly used NRTIs are tenofovir (TDF), zidovudine (ZDV) or stavudine (D4T) in combination with lamivudine (3TC) or emtricitabine (FTC) together with either efavirenz (EFV) or nevirapine (NVP). Boosted protease inhibitors such as lopinavir/ritonavir are used in second–line therapy. When there is evidence to suggest ongoing viral replication (viral load > 1000 copies/ml) in spite of adherence to ART, it is defined as virological failure. Reasons for treatment failure include primary resistance (4) and drug toxicity causing poor adherence(6).

Adherence is an important determinant of whether an individual will develop resistance to their drug regimen. Adherence between 70 – 90% has been thought of as being a driver of drug resistance mutations but Parienti *et al* showed that in individuals with low to moderate adherence, sustained interruption played a

more important role in the development of resistance mutations(7,8). Treatment interruptions may result from logistical or transport problems in resource limited settings(9); and side effects may result in poor adherence to a particular drug(10). Bangsberg *et al* note that some infected individuals experience a decline of adherence over an extended period after all HIV symptoms have resolved(11). Such patterns of poor adherence result in effective inadvertent monotherapy, because certain drugs have a longer half-life (especially the NNRTIs), creating selection pressure for drug-resistant mutant sub-populations of virus (2). Not all individuals experiencing treatment failure develop resistance and the treatment failure may still be corrected with improved adherence: in the PASER cohort 29% of patients failing first-line ART had wild–type virus (12).

Toxicity of the ART drugs is an important reason individuals may struggle with adherence and may result in them stopping their medication altogether. Ammassari *et al* showed that 30% of infected individuals experienced moderate to severe symptoms whilst on ART and this was significantly associated with adherence (13). Side effects vary between drugs and duration on ART and can range from hypersensitivity reactions to lipodystrophy and mitochondrial toxicity.

Mechanisms of ART drug resistance

On a genetic level drug resistance emerges due to single or multiple base changes in the HIV genes encoding for the amino acid sequence of an ART drug target. As a result there is a decreased affinity of the drug for its binding domain on that protein, in the case of NNRTIs and PIs. (6,14). The mechanism for NRTI resistance is either excision or discrimination (of nucleotide building blocks in DNA transcription), and this depends on the individual mutation.

A classic example of a mutation that would affect NRTIs is the M184V mutation commonly associated with resistance to lamivudine and emtricitabine: methionine is replaced by valine at the 184 position of the RT enzyme.

The type of mutation that develops is determined by the class of drugs and the duration of the regimen to which the infected individual is exposed (6). Each drug-resistant mutant can be present within the viral population prior to ART exposure but is outnumbered by the “wild type” virus(6). Poor adherence or interruptions result in “selection pressure” and the multiplication of resistant virus. This is further compounded by the poor “proofreading” capacity of the RT enzyme (6).

In the South African guidelines the infected individual is said to be experiencing virological failure if the viral load is > 1000 copies/ml on two consecutive occasions (done 6-12 weeks apart) 24 weeks after the commencement of ART(15). Which mutation develops first is usually dependent on the genetic barrier of the drugs within the regimen, NNRTIs and lamivudine have the lowest barrier(6). Therefore an individual experiencing treatment failure on the first-line regimen of tenofovir/emtricitabine/efavirenz will typically develop M184V and K103N mutations. These initial mutations are followed by more NNRTI mutations. K65R may occur if they are taking tenofovir. Thymidine analogue mutations (TAMS) occur if the individual is taking AZT, three or more TAMs are needed to significantly decrease susceptibility to several NRTIs(6). Interestingly, K65R and TAM mutations exhibit a negative association and have been shown to phenotypically antagonize each other(16). In other words they do not often occur together and if occurring together the expected resistance profile present towards certain ART drugs decreases (16). Other NRTI mutations which cause wide cross-class resistance are the Q151M and the T69 insertion(15,17). The usage of triple NRTI ART regimens increased the likelihood for the accumulation of TAMs. In the DART trial 55% of patients on triple NRTI therapy developed TAMs by 24 weeks(18). Triple NRTI regimens are no longer considered standard of care.

Much of our understanding regarding HIV resistance has been developed in those infected with HIV-1 subtype B virus in the United States and Europe and extrapolated to HIV-1 subtype C virus which is the predominant subtype in the Southern Africa and India. There is therefore a need to increase the knowledge around HIV drug resistance in HIV subtype C, especially as newer ART drugs become available.

Literature review of studies reporting ART drug resistance in sub-Saharan Africa

The aim of this section of the literature review was to:

- Characterize and compare studies conducted in sub-Saharan Africa with the aim to describe the commonest mutational patterns observed in individuals experiencing failure on first-line ART.
- Describe primary resistance to first-line ART in this setting.
- Describe the impact of virological monitoring on the development of resistance compared to the use of clinical and immunological monitoring criteria.
- Discuss the prevalence of the K65R mutation and its impact on the choice of second-line therapy.
- Discuss the impact of prior single–dose nevirapine on the development of resistance.
- Describe baseline characteristics that contribute to the development of resistance and whether they influence the type of resistance pattern that develops.

The role of drug resistance testing after first-line treatment failure was previously intensely debated. However, two recent clinical trials have shown that a more extensive mutational pattern at NNRTI-based first-line failure was not associated with poorer virological outcomes on second-line that included lopinavir/ritonavir. Indeed in these studies those with more extensive resistance at first-line failure were more likely to achieve virological suppression on second line ART (19,20). This evidence justifies the approach of following standardized treatment regimen sequencing without resistance testing as laid out in the WHO treatment guidelines (6). There may be a role for resistance testing at first-line failure to distinguish those patients with resistance versus those with non-adherence. However, in SA HIV drug resistance testing is generally preserved for individuals experiencing treatment failure on a second-line regimen who have demonstrated good compliance, to determine the best choice of third-line ART regimen. However, in many countries in sub-Saharan Africa resistance testing even for this indication is unaffordable and unavailable. Studies have been undertaken comparing treatment efficacy of boosted PI's in combination with either NRTI's or raltegravir as part of second-line therapy without resistance testing: raltegravir has been shown to be similar to 2 NRTIs in terms of virological outcomes and side effects (19,20). Therefore the efficacy of boosted PIs with NRTIs (even if they are compromised by cross-resistance) argues against the use of genotyping in this clinical context.

The question of outcomes with genotype guided therapy compared with standardized therapy was studied by Lessells *et al*(21). In this study in which standardized treatment recommendations were compared to the guidance ultimately obtained from genotyping, in patients failing first line ART it was found that 71.9% of patients were receiving the appropriate therapy with a standardized approach compared with what would have been prescribed using the recommendation obtained from the specialist physician who interpreted the genotype results. 26% of patients were found to have genotype-guided treatment recommendations that differed from the standardized therapy after treatment failure on first-line ART. Thus there appears to be some potential benefit from genotyping in this setting but this has to be weighed against the cost that has to be borne by the resource limited public sector(21). Cost benefit studies regarding genotyping have unfortunately mainly been limited to the United States and Europe with studies finding genotyping as being a cost effective practice that is beneficial to their respective health systems (22,23). Genotyping may be cost effective compared with the conventional approach of empirically switching to a subsequent generation of ART and that is in the case of resistance to second-line ART and switching to third-line therapy(24). It was suggested that the cost of genotyping was outweighed by the cost of switching a patient to expensive third-line therapy which may be unnecessary if the patient is still treatment sensitive. Their health systems and population demographics however differ from ours therefore the results likely do not translate to our clinical setting with its resource constrains and very large numbers of patients.

Primary or transmitted drug resistance needs to be considered as a cause of first-line ART failure, especially with the high HIV transmission rates in sub-Saharan Africa and large numbers on ART. Hosseinipour *et al* in a WHO drug resistance survey across 12 countries noted baseline resistance in 4,8% (2007) of patients starting first-line therapy and by 2010 it had risen to 6.8% and the increase was driven by NNRTI–

associated mutations(12). In the South African context Manasa *et al.* looked at the trend of primary HIV resistance over a period of ten years between 2000 and 2010 and noted that the prevalence of primary HIV resistance remained relatively low at less than 5% and there was no evidence to suggest an increase in the trend over the ten year period(25). By 2016 Steegen *et al* reported primary resistance in 9% of ART naïve individuals confirming a rise in the prevalence of primary resistance and underscoring the argument for genotype screening of ART-naïve individuals should this become affordable in the future(26). A slight deviation above the norm was noted in 2002, of 6,7%, but thereafter the levels remained consistently below 5%(25). With widespread ongoing transmission of the virus in sub-Saharan Africa, primary resistance cannot be ignored and its impact for patients affected can mean ART failure despite good adherence. Genotyping, pre-ART initiation, and its impact on treatment outcomes has not been reviewed in our clinical setting but has some potential benefits as noted by Oette *et al* where it was revealed that initiation of ART guided by resistance testing for patients with primary resistance resulted in similar ART efficacy to patients being treated who had the wild–type virus(27). This strategy is unlikely to be affordable in sub-Saharan African countries.

The resistance profile that develops in a particular individual and the impact it will have on second- and third-line ART has been shown to be determined by a number of factors. One of the factors determining what the resistance profile that develops at a cohort level is the method of treatment monitoring. Hosseinipour *et al* observed that when using clinical criteria such as the WHO staging and immunological parameters such as the CD4 count to monitor treatment response, this increased patients' susceptibility to the development of not only a higher number of resistance mutations but these patients also develop mutations associated with pan–nucleoside resistance(17). 17% of the patients observed to have ART failure in this study had complex mutational patterns that would likely substantially diminish second-line therapy NRTI effectiveness when the clinical and immunological criteria were used to determine whether a patient was failing first-line therapy(17). The mutations of interest included the Q151M complex(17). This is in sharp contrast to the results of a study conducted by Wallis *et al* that monitored a cohort of patients receiving ART enrolled in the "CIPRA – SA safeguard the household study"(15). These patients were monitored by doing HIV viral load measurements 12 weekly and virological failure was defined as having a viral load >1000 copies/ml or not reducing the viral load by at least log 1.5 at week 12 of treatment(15). Complex mutational patterns were not observed as frequently: thymidine analogue mutations were seen in about 1% of the cohort and Q151M was not observed(15). The main factors here appear to be the method of monitoring (viral load monitoring detects failure sooner than CD4 monitoring) and the increased frequency with which these patients were being monitored compared to Hosseinpours' cohort where it was noted that the average time spent on ART at the time of genotyping was about 36.5 months (17). This underscores the value of standardized, scheduled viral load monitoring as opposed to the use of clinical and immunological parameters to track a patient's response to ART.

With the phasing out of stavudine as part of first-line therapy due to its severe mitochondrial toxicities tenofovir has become part of standard first-line ART in South Africa. While providing a better side effect profile these two drugs share an ability to select out NRTI mutational patterns which may create challenges in the selection of NRTIs in second-line therapy. Tang *et al* showed that failure on stavudine can produce mutational patterns that result in TDF and AZT cross-resistance although it was found that the K65R or K70E mutation that impair TDF efficacy occurred in 5.3% of patients failing therapy and TAMs or Q151M which hamper AZT activity occurred in 22% of individuals(28). This review revealed that TDF was more likely to retain antiviral activity following stavudine failure than AZT (28). In another study done in Gugulethu, Cape Town, 9% of patients who had a resistance test after failing a first-line stavudine regimen had K65R (29). With the move towards TDF as part of the fixed drug combination regimen that includes emtricitabine and efavirenz in first-line there is a growing concern about the emergence of the K65R mutation and the rates at which this mutation arises in patients on tenofovir containing therapy(30–32) . Sunpath *et al* in a retrospective study of a cohort of patients failing TDF based regimens infected with HIV subtype C showed a very high rate for the selection of K65R (69.7%) - a much higher rate than that observed for stavudine in the review by Tang et al(33). With K65R susceptibility to AZT remains, but great challenges emerge when the patient has comorbidities that preclude AZT use. Some reassurance can be taken from the fact that K65R tended to occur in the absence of TAMs likely due to an antagonistic relationship between the two types of mutations(33). Although the change from stavudine to TDF has been

justified in that the side effect profile of the latter is more acceptable therefore decreasing the likelihood of defaulting medication due to poor tolerance, one concern about TDF is that it may more rapidly become resistant when a patient fails first-line ART. Doualla–Bell *et al* showed that the K65R mutation can be selected for by TDF-based ART within 12 weeks of therapy commencement (34).

Single–dose nevirapine (sd-NVP) provided to pregnant women to prevent mother to child transmission of HIV–1 virus was common practice for many years. Data showing that the development of NNRTI resistance in this population is fairly consistent. Cunningham and colleagues revealed an NNRTI mutation prevalence of approximately 15% at 6 – 8 weeks after mothers were given nevirapine to prevent MTCT (35). The impact that this has on later first-line ART and its effect on the further development of resistance has been studied. Wallis *et al* looked at women enrolled in the CIPRA–SA study to evaluate the emergence of complex mutational patterns in patients failing first–line ART. Of the women failing first-line ART a greater proportion had prior exposure to single dose nevirapine (39% vs. 28%)(15). Other studies have shown that these patients are more likely to fail nevirapine-based ART and there is associated mortality in these patients(36). Lockman *et al* demonstrated this in a study in which 241 women with prior exposure to single–dose nevirapine were either started on nevirapine containing combination ART or lopinavir/ritonavir-based ART. More women started on nevirapine based ART reached the primary end point of the study which included virological failure or death (26% vs 8%)(36). Boltz *et al* compared the end points of virologic failure or death in women that had NVP related mutations at study entry with a prior history of single–dose nevirapine and without a history of single-dose nevirapine started on NVP based combination ART(37). Forty-one percent of women in the group with prior exposure to sd-NVP reached the primary endpoints compared with 21% in the group without prior exposure to sd-NVP (37). Interestingly there is evidence to suggest that the nevirapine mutations "fade" beyond a period of 12 months(38). Lockmans' study showed that ritonavir-boosted lopinavir based therapy lost its advantage over nevirapine as the interval since single–dose nevirapine treatment and the commencement of ART increased(36). Therefore it may be fair to conclude that prior use of single–dose nevirapine has an effect on the choice of future first-line ART though the effect is greatest within the first 12 months. The use of this strategy is now no longer advised, but there are women who had previous single-dose nevirapine who will still need to start ART. Thus the impact of single-dose nevirapine still needs to be considered.

Nevirapine not only causes NNRTI associated mutations of concern but also causes so called "connection domain mutations" (CDMs) on RT and there is interest in N348I and its effect on second-line NNRTIs namely etravirine(14). The studies used to determine the frequency of this particular mutation used stavudine and lamivudine as their NRTI backbones with efavirenz being compared to nevirapine(14). The frequency of the CDMs was higher in the nevaripine cohort – 45% compared to the efavirenz cohort – 12%(39). Its effect on etravirine however seems to depend on the presence of added mutations as the presence of N348I in isolation does not reduce etravirine activity significantly(14).

Determining which variables are associated with the development of resistance has been a focus of many studies. Age, gender, employment, recent opportunistic infection, CD4 count at the time of enrollment, plasma HIV viral load at the time of enrollment and WHO stage are some of the variables that have been considered(40). Marconi *et al* found that patients with viral loads of between 5000 – 99,999 copies/ml at initial recruitment were more likely to develop mutations. This is in contrast to Wallis *et al* who found that the pre-treatment viral load did not have a significant relationship with virological failure and that subjects who failed were more likely to have low CD4 cell counts(15). The other variables listed above did not seem to be associated with treatment failure in a statistically significant way in that study. Another variable that may be considered to influence the development of resistance is the prior use of suboptimal antiretroviral therapy. One would expect that when single or dual–therapy regimens are used that this would generate mutations to those drugs due to the limited viral suppression provided by the chosen agents, therefore having an impact on subsequent regimens, but not all studies have confirmed this (40).

Optimal drug adherence ensures viral load suppression and helps ensure clinical response to ART. The exact level of adherence needed to prevent the development of drug resistance has been a topic of research with some of the earlier studies with protease inhibitors suggesting a requirement >95%(41–43). There are however some challenges to this research. The way in which one measures adherence can be inaccurate

especially in a resource limited setting where patient follow–up is already challenging. Some of the methods used in studies include having patients answer adherence questionnaires and working out cumulative adherence via formulas, pill count, prescription refill data and plasma drug concentrations(7,15,44).

Not all of these methods are available in the resource-constrained public sector as most of the studies utilizing these above methods were conducted outside of Africa. Evidence suggests that adherence between 70 – 90%(7) usually results in the development of resistance but there are some studies that do not reveal a statistically significant relationship between adherence and the development of resistance as was demonstrated by Marconi *et al*(40). This figure of 70 – 90% is probably outdated in that it applies to earlier ART regimens used in the 1990s and different drugs have individual adherence–resistance relationships (i.e. the range of adherence that is required to select out a resistance mutation). For example, Bangsberg *et al* showed that NNRTI resistance mutations develop in an adherence range of between 2 and 60% and viral suppression may be observed at adherence levels above 60% when NNRTI-based regimens are considered(11).

In summary, HIV drug resistance testing may have benefit in terms of ART management decisions, but cannot be justified in the resource limited public health sector at first-line ART failure on account of the cost that this investigation incurs. There has been evidence to show that continued use of NRTI based therapy with a boosted PI in patients with first-line ART failure compares favorably with the use of the integrase inhibitor raltegravir when used in place of the NRTI backbone in achieving satisfactory viral load suppression(19,20). Within the private health care setting where it may be affordable it allows for a more targeted approach to the management of individuals experiencing first-line treatment failure. My MMed study describes the resistance mutation patterns observed in the private sector in South Africa after first-line ART failure and gives us an opportunity to compare our results with studies done in the public sector. There may be important differences observed that require further exploration. The development of resistance to ART agents is a topic that cannot be ignored and will ultimately become a public health problem resulting in an increase in the burden on an already strained health care system. The limited choice of ART agents available for use in the public health care system in sub-Saharan Africa provides a compelling reason to ensure that best practice is at the forefront in terms of adherence counseling and drug choices and supply thereby allowing us to preserve more costly second- and third-line ART agents.

As is evident from this review, studies characterizing drug resistance mutations detected when individuals experience first-line failure in sub-Saharan Africa have mainly been conducted in the public sector. Few, if any, studies have been done focused on drug resistance patterns in the private sector where genotyping is more frequently done in clinical practice and data regarding patient characteristics, comorbidities and relevant drug history is accessible. The study that I conducted for my MMed of drug resistance in a private sector disease management programme in South Africa thus fills a knowledge gap.

References

1. HIV/AIDS W. WHO | HIV/AIDS. World Health Organization; Available from: http://www.who.int/mediacentre/factsheets/fs360/en/
2. UNAIDS.org. Available from: http://www.unaids.org/
3. aidsinfo.unaids.org. Available from: http://aidsinfo.unaids.org/
4. Longo, Fauci, Kasper, Hauser, Jameson, Loscalzo. Harrison's Principles of Internal Medicine. 18th ed. McGraw-Hill;
5. Kumar, Abbas, Fausto, Aster. Robbins and Cotran Pathologic Basis of Disease. 8th ed. Saunders; 2009.
6. Meintjes G. Antiretroviral resistance. Continuing Medical Education 2008 p. 230.
7. Sethi, Ajay K.Celentano, David D.Gange, Stephen J.Moore, Richard D.1, 2Gallant, Joel E.1 2. Association between Adherence to Antiretroviral Therapy and Human Immunodeficiency Virus Drug Resistance.
8. Parienti J-J, Das-Douglas M, Massari V, Guzman D, Deeks SG, Verdon R, et al. Not all missed doses are the same: sustained NNRTI treatment interruptions predict HIV rebound at low-to-moderate adherence levels. PLoS One. Public Library of Science; 2008 Jul 30 ;3(7):e2783.
9. Mills EJ, Nachega JB, Bangsberg DR, Singh S, Rachlis B, Wu P, et al. Adherence to HAART: a systematic review of developed and developing nation patient-reported barriers and facilitators. PLoS Med. 2006 Nov;3(11):e438.
10. Gardner EM, Burman WJ, Maravi ME, Davidson AJ. Selective drug taking during combination antiretroviral therapy in an unselected clinic population. J Acquir Immune Defic Syndr. 2005 Nov 1;40(3):294–300.
11. Bangsberg DR, Kroetz DL, Deeks SG. Adherence-resistance relationships to combination HIV antiretroviral therapy. Curr HIV/AIDS Rep. 2007 Apr 26;4(2):65–72.
12. Hosseinipour MC, Gupta RK, Van Zyl G, Eron JJ, Nachega JB. Emergence of HIV drug resistance during first- and second-line antiretroviral therapy in resource-limited settings. J Infect Dis. 2013 Jun 15;207 Suppl:S49-56.
13. Ammassari A. Self-reported symptoms and medication side effects influence adherence to highly active antiretroviral therapy in persons with HIV infection. J Acquir Immune Defic Syndr. 2001;28(5).
14. Gupta S, Vingerhoets J, Fransen S, Tambuyzer L, Azijn H, Frantzell A, et al. Connection domain mutations in HIV-1 reverse transcriptase do not impact etravirine susceptibility and virologic responses to etravirine-containing regimens. Antimicrob Agents Chemother. 2011 Jun;55(6):2872–9.
15. Wallis CL, Papathanasopolous MA, Fox M, Conradie F, Ive P, Orrell C, et al. Low rates of nucleoside reverse transcriptase inhibitor resistance in a well-monitored cohort in South Africa on antiretroviral therapy. Antivir Ther. NIH Public Access; 2012 Jan;17(2):313–20.
16. Parikh UM, Bacheler L, Koontz D, Mellors JW. The K65R mutation in human immunodeficiency virus type 1 reverse transcriptase exhibits bidirectional phenotypic antagonism with thymidine analog mutations. J Virol. 2006 May;80(10):4971–7.
17. Hosseinipour MC, van Oosterhout JJG, Weigel R, Phiri S, Kamwendo D, Parkin N, et al. The public health approach to identify antiretroviral therapy failure: high-level nucleoside reverse transcriptase inhibitor resistance among Malawians failing first-line antiretroviral therapy. AIDS. NIH Public Access; 2009 Jun 1;23(9):1127–34.
18. Virological response to a triple nucleoside/nucleotide analogue regimen over 48 weeks in HIV-1-infected adults in Africa. AIDS, 2006 Jun 26;20(10):1391–9.
19. Paton NI, Kityo C, Hoppe A, Reid A, Kambugu A, Lugemwa A, et al. Assessment of second-line antiretroviral regimens for HIV therapy in Africa. N Engl J Med. 2014 Jul 17;371(3):234–47.
20. La Rosa AM, Harrison LJ, Taiwo B, Wallis CL, Zheng L, Kim P, et al. Raltegravir in second-line antiretroviral therapy in resource-limited settings (SELECT): a randomised, phase 3, non-inferiority study. Lancet HIV. 2016 Jun;3(6):e247-58.
21. Lessells RJ, Stott KE, Manasa J, Naidu KK, Skingsley A, Rossouw T, et al. Implementing

antiretroviral resistance testing in a primary health care HIV treatment programme in rural KwaZulu-Natal, South Africa: early experiences, achievements and challenges. BMC Health Serv Res. 2014 Jan;14:116.
22. Corzillius M, Mühlberger N, Sroczynski G, Jaeger H, Wasem J, Siebert U. Cost effectiveness analysis of routine use of genotypic antiretroviral resistance testing after failure of antiretroviral treatment for HIV. Antivir Ther. 2004 Feb;9(1):27–36.
23. Sendi P, Günthard HF, Simcock M, Ledergerber B, Schüpbach J, Battegay M. Cost-effectiveness of genotypic antiretroviral resistance testing in HIV-infected patients with treatment failure. PLoS One. 2007 Jan;2(1):e173.
24. Lorenzana SB, Hughes MD, Grinsztejn B, Collier AC, Luz PM, Freedberg KA, et al. Genotype assays and third-line ART in resource-limited settings: a simulation and cost-effectiveness analysis of a planned clinical trial. AIDS. 2012 Jun 1;26(9):1083–93.
25. Manasa J, Katzenstein D, Cassol S, Newell M-L, de Oliveira T. Primary drug resistance in South Africa: data from 10 years of surveys. AIDS Res Hum Retroviruses. 2012 Jun;28(6):558–65.
26. Steegen K, Carmona S, Bronze M, Papathanasopoulos MA, van Zyl G, Goedhals D, et al. Moderate Levels of Pre-Treatment HIV-1 Antiretroviral Drug Resistance Detected in the First South African National Survey. Blackard J, editor. PLoS One. 2016 Dec 1;11(12):e0166305.
27. Oette M, Kaiser R, Däumer M, Petch R, Fätkenheuer G, Carls H, et al. Primary HIV drug resistance and efficacy of first-line antiretroviral therapy guided by resistance testing. J Acquir Immune Defic Syndr. 2006 Apr 15;41(5):573–81.
28. Tang MW, Rhee S-Y, Bertagnolio S, Ford N, Holmes S, Sigaloff KC, et al. Nucleoside reverse transcriptase inhibitor resistance mutations associated with first-line stavudine-containing antiretroviral therapy: programmatic implications for countries phasing out stavudine. J Infect Dis. 2013 Jun 15;207 Suppl:S70-7.
29. Orrell C, Walensky RP, Losina E, Pitt J, Freedberg KA, Wood R. HIV type-1 clade C resistance genotypes in treatment-naive patients and after first virological failure in a large community antiretroviral therapy programme. Antivir Ther. 2009 Jan;14(4):523–31.
30. Skhosana L, Steegen K, Bronze M, Lukhwareni A, Letsoalo E, Papathanasopoulos MA, et al. High prevalence of the K65R mutation in HIV-1 subtype C infected patients failing tenofovir-based first-line regimens in South Africa. Maga G, editor. PLoS One. 2015 Feb 6 ;10(2):e0118145.
31. Hoffmann CJ, Ledwaba J, Li J-F, Johnston V, Hunt G, Fielding KL, et al. Resistance to tenofovir-based regimens during treatment failure of subtype C HIV-1 in South Africa. Antivir Ther. NIH Public Access; 2013;18(7):915–20.
32. Van Zyl GU, Liu TF, Claassen M, Engelbrecht S, de Oliveira T, Preiser W, et al. Trends in Genotypic HIV-1 Antiretroviral Resistance between 2006 and 2012 in South African Patients Receiving First- and Second-Line Antiretroviral Treatment Regimens. Landay A, editor. PLoS One. Public Library of Science; 2013 Jun 26;8(6):e67188.
33. Sunpath H, Wu B, Gordon M, Hampton J, Johnson B, Moosa M-YS, et al. High rate of K65R for antiretroviral therapy-naive patients with subtype C HIV infection failing a tenofovir-containing first-line regimen. AIDS. 2012 Aug 24;26(13):1679–84.
34. Doualla-Bell F, Avalos A, Brenner B, Gaolathe T, Mine M, Gaseitsiwe S, et al. High prevalence of the K65R mutation in human immunodeficiency virus type 1 subtype C isolates from infected patients in Botswana treated with didanosine-based regimens. Antimicrob Agents Chemother. 2006 Dec;50(12):4182–5.
35. Cunningham CK, Chaix M-L, Rekacewicz C, Britto P, Rouzioux C, Gelber RD, et al. Development of resistance mutations in women receiving standard antiretroviral therapy who received intrapartum nevirapine to prevent perinatal human immunodeficiency virus type 1 transmission: a substudy of pediatric AIDS clinical trials group protocol 3. J Infect Dis. 2002 Jul 15;186(2):181–8.
36. Lockman S, Hughes MD, McIntyre J, Zheng Y, Chipato T, Conradie F, et al. Antiretroviral therapies in women after single-dose nevirapine exposure. N Engl J Med. 2010 Oct 14;363(16):1499–509.
37. Boltz VF, Bao Y, Lockman S, Halvas EK, Kearney MF, McIntyre JA, et al. Low-Frequency

Nevirapine (NVP)-Resistant HIV-1 Variants Are Not Associated With Failure of Antiretroviral Therapy in Women Without Prior Exposure to Single-Dose NVP. J Infect Dis. 2014 Jan 16;209(5):703–10.
38. Eshleman SH, Mracna M, Guay LA, Deseyve M, Cunningham S, Mirochnick M, et al. Selection and fading of resistance mutations in women and infants receiving nevirapine to prevent HIV-1 vertical transmission (HIVNET 012). AIDS. 2001 Oct 19;15(15):1951–7.
39. Brehm JH, Koontz DL, Wallis CL, Shutt KA, Sanne I, Wood R, et al. Frequent emergence of N348I in HIV-1 subtype C reverse transcriptase with failure of initial therapy reduces susceptibility to reverse-transcriptase inhibitors. Clin Infect Dis. 2012 Sep;55(5):737–45.
40. Marconi VC, Sunpath H, Lu Z, Gordon M, Koranteng-Apeagyei K, Hampton J, et al. Prevalence of HIV-1 drug resistance after failure of a first highly active antiretroviral therapy regimen in KwaZulu Natal, South Africa. Clin Infect Dis. 2008 May 15;46(10):1589–97.
41. Paterson DLSS. Adherence to Protease Inhibitor Therapy and Outcomes in Patients with HIV Infection.
42. Bangsberg DR FH, Charlebois E, Zolopa A, Holodniy M, Sheiner L, Bamberger J, et al. Adherence to protease inhibitors, HIV-1 viral load, and development of drug resistance in an indigent population. AIDS. 2000;14(4):357–66.
43. Arnsten JH, Demas PA, Farzadegan H, Grant RW, Gourevitch MN, Chang CJ, et al. Antiretroviral therapy adherence and viral suppression in HIV-infected drug users: comparison of self-report and electronic monitoring. Clin Infect Dis. 2001 Oct 15;33(8):1417–23.
44. Harrigan, P. Richard1, 2Hogg, Robert S.1, 2Dong, Winnie W. Y.Yip, BenitaWynhoven, BrianWoodward, JustinBrumme, Chanson J.Brumme, Zabrina L.1, 2Mo, TheresaAlexander, Chris S.Montaner, Julio S. G.1 2. Predictors of HIV Drug-Resistance Mutations in a Large Antiretroviral-Naive Cohort Initiating Triple Antiretroviral Therapy.
45. LET OUR ACTIONS COUNT. 2012;2017–22. Available from: http://sanac.org.za/wp-content/uploads/2017/01/Final-NSP-Document.pdf
46. Wallis CL, Mellors JW, Venter WDF, Sanne I, Stevens W. Varied patterns of HIV-1 drug resistance on failing first-line antiretroviral therapy in South Africa. J Acquir Immune Defic Syndr. 2010 Apr 1;53(4):480–4.
47. Meintjes G. Antiretroviral resistance. Emergence of resistance. 2005;23(5).
48. Nachega JB, Hislop M, Dowdy DW, Gallant JE, Chaisson RE, Regensberg L, et al. Efavirenz versus Nevirapine-based Initial Treatment of HIV Infection: Clinical and Virological Outcomes in Southern African Adults. AIDS Oct. 2008;18(2216):2117–25.
49. Hosseinipour M, Kumwenda J, Weigel R, Brown L, Mzinganjira D, Mhango B, et al. Second-line treatment in the Malawi antiretroviral programme: high early mortality, but good outcomes in survivors, despite extensive drug resistance at baseline. HIV Med. Blackwell Publishing Ltd; 2010 Mar;11(8):510–8.
50. Kuritzkes DR, Bassett RL, Hazelwood JD, Barrett H, Rhodes RA, Young RK, et al. Rate of thymidine analogue resistance mutation accumulation with zidovudine- or stavudine-based regimens. J Acquir Immune Defic Syndr. 2004 May 1;36(1):600–3.
51. Lockman S, Hughes MD, McIntyre J, Zheng Y, Chipato T, Conradie F, et al. Antiretroviral therapies in women after single-dose nevirapine exposure. N Engl J Med. NIH Public Access; 2010 Oct 14;363(16):1499–509.

Characterization of and risk factors for HIV-1 resistance mutations following first line antiretroviral failure in the Southern African private sector

Authors:
Edgar Lutaaya[1]
Michael Hislop[2]
Kathryn Manning[1]
Liezel Dunn[2]
*Carole Wallis[3]
*Graeme Meintjes[1]

Affiliations:
1. University of Cape Town
2. Aid for AIDS
3. Lancet Laboratories

*Joint senior authors

Corresponding author:
Dr Edgar Lutaaya
Department of Medicine
University of Cape Town
Groote Schuur Hospital
Observatory
7925
South Africa
Phone: +27-722256749
Email: edgarlutaaya@yahoo.com

Word Count:
Abstract: 457
Manuscript: 4089
References: 56
Tables: 7
Figures: 8

Key words: Human immunodeficiency virus, HIV, antiretroviral therapy, ART, resistance, mutations.

Abstract

Background: First-line antiretroviral therapy (ART) regimen choices have evolved over the past 15 years in South Africa. Many patients develop HIV drug resistance mutations when they fail first-line ART. The effect of different drug regimens and other patient related factors on drug resistant mutations selected at first line failure are not well characterised in Southern Africa with its predominantly subtype C epidemic.

Objectives: To characterize HIV resistance mutations in patients failing first-line ART in the private sector in Southern Africa, and risk factors associated with resistance and particular resistance mutations.

Methods: This was a retrospective observational study linking two databases. One database was that of the Aid for AIDS (AfA) disease management programme (for clinical and ART regimen data) and the other was that of Lancet Laboratories where HIV genotypic sequence data were stored. Variables included in analyses were age, gender, province, WHO stage at time of resistance testing (HIVDR), duration of ART at HIVDR, prior monotherapy or dual therapy, viral load and CD4 count prior to starting ART, viral load and CD4 count at HIVDR, duration that the viral load was >400 copies/ml prior to HIVDR and HIV subtype. Data on patients who had a resistance test between 2008 and 2014 while failing first line ART was extracted from these databases. Fisher's exact test and Chi-squared test were used to analyse categorical variables and Wilcoxon rank sum test for continuous variables. For multivariate analyses, logistic regression models were used.

Results: Patients who registered with AfA between 1998 and 2013 and had a resistance test performed whilst experiencing viral failure on a non-nucleoside reverse transcriptase inhibitor (NNRTI) regimen with no history of prior failure on a protease inhibitor (PI) regimen were included: 265 (95.3%) patients had a reverse transcriptase mutation of any kind, 253 (91%) had an NNRTI mutation and 246 (88.5%) had a nucleoside reverse transcriptase inhibitor (NRTI) mutation (n=278). The commonest mutation was the M184V mutation (80.6%), 44.2% had at least one thymidine analogue mutation (TAM) and 42.1% had the K103N/S mutation. Notably there was a median of 18 months during which the viral load was not suppressed prior to the resistance tests, which likely contributed to the high prevalence of mutations. A total of 83 (29.9%) patients were found to have the K65R mutation, 72 of these patients were on tenofovir (TDF) at the time of resistance testing demonstrating the strong association between TDF and the K65R mutation.

Conclusions: The mutational patterns observed in our study and their prevalence were similar to those noted in previous studies done within the region's public sector. There was a high prevalence of the K65R mutation in patients failing TDF-containing regimens. A longer duration on ART and failing ART were correlated with an increased number of TAMs.

Introduction

Antiretroviral therapy (ART) first became available in the South African private sector in the late 1990s and in the public sector in 2004. An increasing number of individuals have been started on ART such that South Africa now has the highest number of people on ART in the world: an estimated 3 million in the public sector have started ART and several hundred thousand in the private sector (45). As a result, the emergence of ART resistance has become one of the major concerns. Amongst the challenges that face South Africa, with its resource limitations in terms of health care provision, is finding cost effective ways to prevent ART resistance and if it occurs to detect it early and change treatment appropriately.

In South Africa, virological failure on ART is defined as an HIV viral load of >1000 copies/ml on at least two separate occasions more than 24 weeks after ART initiation or a drop in viral load <log 1.5 from baseline at 12 weeks post initiation (15). The development of resistance results in an inability of the drugs to inhibit the replication of the human immunodeficiency virus (HIV) and it then becomes necessary to change to a new ART regimen without significant cross-resistance in order to avoid both immunological and clinical decline.

Resistance mutations are usually a result of a single nucleotide substitutions in a particular region of the gene encoding an HIV protein. These amino acid changes result in a structural or charge change to the protein which interferes with ART drug binding or action. The commonest HIV resistance mutations described include the M184V mutation associated with lamivudine, K103N as well as Y181C mutations associated with NNRTI failure and the thymidine analogue mutations (TAMs) selected for by zidovudine (AZT) and stavudine (D4T) namely: M41L, D67N, L210W, K70R,T215Y/F, and K219Q/E. Of concern is the emergence of complex mutational patterns which allow for resistance across a broad spectrum of drugs within a class and may have implications for the choice of drugs in second-line therapy. These include the K65R, Q151M, T69 insertion and the presence of more than three TAMs (46).

Adherence in the range between 70-90% has been shown to contribute to the development of resistance. Excellent adherence has been shown to be the most important preventer of resistance development (12,47). There is an opportunity to describe connections between ART drugs used in first-line regimens and the resultant mutational patterns when the regimen fails in the private sector in Southern Africa because access to resources for genotypic resistance testing has allowed for this testing. Analyzing this data will potentially broaden our understanding of the mutational patterns occurring in ART programmes within subtype C epidemics and inform on the utility of NRTI drugs and NNRTI drugs in subsequent lines of therapy. Second-generation NNRTI drugs (rilpivirine and etravirine) may be considered in third line ART regimens when there is extensive resistance to other ART classes.

Our primary aim was to characterize the frequency and the patterns of genotypic resistance in the private sector in South Africa among individuals experiencing first-line ART failure. Secondary objectives included determining clinical and treatment factors associated with resistance and particular mutations. A specific focus was to evaluate which baseline and on treatment factors were associated with the development of the K65R mutation.

Methods

Study Design

Our study included individuals who started ART between 1998 and 2013, and had a genotypic resistance test done between 2008 to 2014. It was a retrospective observational study linking two database sources. The one source was from the Aid for AIDS (AfA) disease management programme which included clinical and demographic data from individuals who received ART in the private sector within their programme. The second database was that of the Lancet Laboratories where samples from certain individuals registered with AfA were sent for resistance testing when the individuals were found to be failing an ART regimen. These two databases were linked to address the study aims. The study included all AfA registered individuals failing a first-line NNRTI-based ART regimen who had a resistance test performed by Lancet Laboratories. Adult and paediatric patients were included.

The following information was extracted from the AfA clinical database: age, gender, province of residence, baseline CD4 count and CD4 count at resistance test, baseline viral load and viral load at resistance test, duration on ART at resistance test, first line drugs individuals had been exposed to including changes made as well as the duration taking drugs, the duration of documented unsuppressed viral loads (viral load > 400 copies/ml) prior to resistance testing, and baseline World Health Organization (WHO) stage. The viral subtype and resistance-associated NRTI and NNRTI resistance mutations were accessed from the Lancet Laboratory database. The 2014 IAS-USA HIV drug resistance list was used to define significant resistance mutations associated with NRTIs, nevirapine (NVP) and efavirenz (EFV) that were then recorded for these analyses. All data was de-identified after linking and analyses were performed on the de-identified data.

Aid for AIDS (AfA)Disease Management Programme

AfA was launched in May 1998 and is a private sector HIV disease management programme that provides clinical interventions coupled with clinical and patient support and on-going adherence management for patients on medical aid schemes and corporate treatment programmes in Southern African. The aim of the programme is to partner with healthcare providers and healthcare funders to optimize clinical outcomes by ensuring access to effective ART and regular monitoring of outcomes and potential toxicity, thus limiting the frequency of treatment failure due to poor adherence or adverse effects. Over 78,000 patients are currently registered (more than 272,000 patients have been registered cumulatively to date).

The AfA Clinical Guidelines are regularly revised and updated by the expert consultants on the AfA Clinical Advisory Committee in the light of new developments in HIV management, as a result of the availability of new drugs and tests and changes in starting criteria. Doctors are required to prescribe treatment in line with the AfA guidelines but may motivate for an individualized approach where indicated.

Patients need to be registered on the Aid for AIDS (AfA) programme to have ART medication and blood results paid from their HIV benefit. The treating doctor can complete an application form or call to telephonically register their patient. During registration the doctor will provide relevant blood results, clinical staging information and previous treatment history, where applicable. This data is saved on the AfA database. Once registered, AfA authorize an appropriate ART regimen in discussion with the patient's doctor and assign

relevant monitoring follow-ups for pathology testing.

Monitoring results (for example CD4 count and HIV viral load) may be received from the doctor, submitted electronically to AfA via a file transfer process or AfA may contact the laboratory directly to receive the results. All changes to drug regimens are recorded as well as the reason for the change. A comprehensive note is made with each intervention on the file. All data is loaded on the AfA electronic database.

Although the AfA operates in the private sector, its guidelines are similar to the WHO guidelines with a standardized approach to first-line (NNRTI-based) and second-line (protease inhibitor-based) ART regimens. Important differences between AfA and the public sector ART programme in South Africa are: (i) the AfA programme started in 1998 whereas the public sector programme started in 2004; (ii) in AfA some patients received initial ART with monotherapy or dual NRTIs for affordability reasons in the late 1990s; (iii) certain drugs were available in the private sector before the public sector; and (iv) resistance testing has been available for patients failing ART in AfA since 2001.

Resistance testing at Lancet laboratories

HIV drug resistance testing is performed at Lancet Laboratories using a laboratory developed assay. Briefly, viral RNA is extracted from plasma and amplified to include the full protease and partial reverse transcriptase regions. These regions are sequenced using population based sequencing platform. HIV drug resistance mutations are detected using the Stanford database (https://hivdb.stanford.edu) and the subtype defined using the Rega HIV subtyping tool (http://www.bioafrica.net/rega-genotype/html/subtypinghiv.html).

Statistical analyses

For univariate analysis of categorical variable Fisher's exact test and Chi squared tests were used and Wilcoxon rank sum test was used for continuous variables. Spearman rank-order correlation coefficient was used to analyse the strength and direction of associations between ordinal and continuous variables. As presence of mutations were very common, log-binomial regression analysis was performed using Stata 13 (StataCorp, College Station, TX), with all variables hypothesised to be associated with outcome included in the models. Log transformation was applied to variables with highly skewed distributions. Risk ratios (RR) were presented together with 95% confidence intervals (CI). Adjusted RRs were not estimated in multivariable models that failed to converge. A p-value < 0.05 was regarded as significant.

Ethical approval and consent

This was obtained from the UCT Human Research Ethics Committee (HREC REF 380/2014 and 112/2013). Upon entering the AfA programme patients sign consent to have their data analyzed for programme evaluation and research. The wording on the registration form that the patients sign is: *"I herewith authorize AfA and its agents/medical staff to disclose the medical information relevant to my HIV infection to third parties for the purpose of scientific, epidemiological and/or financial analysis without disclosure of my identity*." The UCT HREC specifically provided permission for this retrospective observational study to be performed linking the AfA and Lancet Laboratories databases provided that the patient data was de-identified prior to analysis.

Results

The study included 278 individuals registered with AfA who had a resistance test performed at Lancet laboratories at the time they were failing an NNRTI regimen, without prior exposure to a PI regimen. These individuals started ART between 1998 and 2013. The resistance tests were performed between 2008 and 2014.

The median age was 37 years (interquartile range (IQR) = 28-43) (Table 1). The majority were over the age of 18 years (n=225; 80.9%). Of the 278 patients, 172 were female (61.9%). The majority of individuals resided in KwaZulu Natal (43.0%) and Gauteng Province (28.5%). World Health Organization (WHO) stage at ART initiation was available for 131 patients: two-thirds were stage 3 or 4. Of the 226 patients for whom this information was available, 9 (4%) had been historically exposed to ART monotherapy and 27 (12%) had been historically exposed to ART dual therapy. Patients had been on ART for a median of 29 months (IQR=15-53) at the time of resistance test. The common NRTIs that patients were receiving were TDF (50.0%), emtricitabine (FTC) (49.6%), lamivudine (3TC) (44.2%), and AZT (31.7%). For the NNRTIs, 81.7% of patients were receiving EFV and 18.4% were receiving NVP. In terms of historical exposure to NRTIs, 63.3% patients had ever received 3TC, 54.9% TDF, 54.9% FTC, 46.0% AZT, 23.9% D4T, 10.2% didanosine (DDI) and 8.9% abacavir (ABC).

The HIV viral load prior to ART was a median of 235,000 copies/ml and CD4 count prior to ART was a median of 78 cells/mm^3 (data for these two data points was missing for 47 patients). At the time of resistance testing median HIV viral load 85,688 copies/ml (data missing for 15) and median CD4 count was 115 cells/mm^3 (data missing for 14). The HIV viral load had been documented to be unsuppressed (greater than 400 copies/ml) for a median of 18 months (IQR = 11-34) at the time of resistance testing. HIV subtype C was the predominant subtype (98.6%).

Frequency of resistance mutations

Of the 278 patients, 265 (95.3%) had any reverse transcriptase resistance mutation (Table 2): 246 (88.5%) patients had at least one NRTI mutation and 253 (91%) had at least one NNRTI mutation. At least one thymidine analogues mutation (TAM) was present for 123 (44.2%) of the patients and the K65R mutation was present for 83 (29.9%) patients. Frequency of individual mutations is shown in Figure 1 and 2. The number of NNRTI mutations and TAMs are shown in Figures 3 and 4 respectively.

Influence of drug exposure to TDF on mutations detected

A total of 150 (54%) patients ever exposed to TDF were compared with 128 (46%) never unexposed to TDF and evaluated for the presence of associated mutations in each group (Table 3). NRTI mutations were found in 134 vs. 112 patients and there was no statistically significant difference (89.3% vs. 87.5% p=0.633). For the NRTI associated mutations the M184V/I mutation was the most prevalent, 128 vs. 96 patients (85.3% vs. 75.0%, p=0.03). As expected the K65R mutation was more common in those who had exposure to TDF, present in 72 vs. 11 patients (48% vs. 8.6%, $p < 0.001$). The K70E mutation (another mutation known to be associated with TDF) occurred in 27 vs. 1 patient (18% vs. 0.78%, $p < 0.001$). TAMs occurred less frequently in the TDF exposed group 49 vs. 74 patients (32.7% vs. 57.8% $p < 0.001$). The commonest TAM occurring in the TDF exposed group being D67N, 28 vs. 38 (18.7 vs. 29.7, p=0.031). The T215Y/F was the commonest TAM found in the TDF unexposed group, 10 vs. 47 (6.7% vs. 36.7%, $p < 0.001$).

It was found that 137 vs. 116 patients (91.3% vs. 90.6%, p=0.837) had NNRTI mutations. There was a statistically significant different difference between groups for 2 mutations: the V106A/M mutation was more common in the group exposed (n=65; 43.3%) to TDF compared to those that had had no TDF exposure (n=40; 31.3%; p=0.038). Similarly, the L100I mutation, although rare, was more common in those exposed to TDF (10.7% vs. 3.1%, p=0.019).

NRTI drugs at resistance test and common mutations

The most frequently used first NRTI by patients at the time of resistance testing was TDF followed by AZT and D4T (n=139 (50%), n=88 (31.7%) and n=26 (9.4%) respectively) (Table 4). The M184V mutation was the commonest NRTI mutation across the all categories (p=0.037); occurring in 119 (85.6%), 66 (75%), 17 (65.4%) and 22 (88%) of TDF, AZT, D4T and other drug exposed groups respectively. The K65R mutation showed a predilection for occurring with TDF exposure at time of resistance test occurring in 72 (52%) of the TDF exposed patients. It was far less common in the other groups occurring in 5 (5.7%), 2 (7.7%) and 4 (16%) of the patients exposed to AZT, D4T and other ART drugs respectively. TAMs occurred frequently in the AZT exposure group 57 (64.8%) and were also noted in 16 (61.5%), 44 (31.7%) and 6 (24%) patients exposed to D4T, TDF and other ART drugs respectively. For the majority of patients, the second drug at resistance test was 3TC or FTC; these were not included in this analysis.

Risk factors for TAMs

Risk factors for TAMs were analysed using univariate analysis (Supplementary table 1). The multivariate model could not be run due to non-convergence. Variables that were found to be significantly associated with presence of one or more TAM on the resistance test included duration on ART (RR=2.32, 95%CI 1.69 – 3.17, p < 0.001), duration failing ART (RR=1.60, 95%CI 1.18 – 2.16, p=0.002), being on DDI at resistance test (RR=1.70, 95%CI 1.33 – 2.16, p <0.001) and any exposure to DDI (RR=2.05, 95%CI 1.63 – 2.58, p<0.001), AZT (RR=1.89, 95%CI 1.44 – 2.5, p<0.001), D4T (RR=1.46, 95%CI 1.13 – 1.90, p=0.004) as well as 3TC (RR=1.42, 95%CI 1.05 – 1.93, p=0.023).

Risk factors for K65R mutation

In the univariate analysis of risk factors for the presence of the K65R mutation (Supplementary table 2) unsurprisingly TDF was the most significant factor (RR=9.12, 95%CI 3.83 – 21.68, p<0.001). The presence of FTC in the drug regimen was also associated (RR=5.75, 95%CI 3.20 – 10.34, p<0.001). Being older than 18 years of age was also a risk factor (RR=3.70, 95%CI 1.24 – 1.02, p=0.019). On multivariate analysis the presence of TDF (RR=30.54, 95%CI 8.15 – 114.5, p<0.001) and NVP (RR=1.59, 95%CI 1.17 – 2.17, p=0.003) in the regimen were significantly associated with the presence of the K65R mutation. Interestingly, the presence of FTC in the regimen appeared protective after adjustment.

Risk factors for NNRTI resistance mutations

In univariate analysis only ever being exposed to NVP (RR=1.08, 95%CI 1.01 – 1.15, p=0.027) was found to be significantly associated with NNRTI mutations (Supplementary table 3). The multivariate model could not be run due to non-convergence.

Frequency of mutations in relation to duration on ART and failing ART

There was a statistically significant correlation between duration on ART and number of

TAMs (Supplementary figure 1) and between duration failing ART and number of TAMs (Supplementary figure 2), although in both instances the strength of the correlation was weak ($r=0.293$ and $r=0.231$ respectively). There was no significant correlation between duration on ART or duration failing ART and number of NNRTI mutations on the resistance test (Supplementary figures 3 and 4).

Discussion

Of the patients included in this study, 95.3% were found to have at least one resistance mutation and of these 88.5% were found to have one or more NRTI mutation and 91% were found to have one or more NNRTI mutation. Of importance 44.2% of the patients were found to have TAMs and 29.9% had the K65R mutation. These results were noted to be consistent with studies done in the public sectors of sub-Saharan Africa (15,17,40). In our study the M184V (80.6%) and the K103N/S (42.1%) mutations occurred prominently.

The frequency with which K65R was present was strongly related to the drug regimen the patient was on at the time of resistance testing – occurring mainly with TDF. Tang *et al* analysed 35 studies from various countries around the world of which 65% were from sub-Saharan Africa; they found the K65R mutation in 6.2% of patients on a D4T based regimen contrasted with Sunpath *et al*, whose study population from KwaZulu Natal, found the same mutation in 69.7% of the cohort who were initiated on a TDF containing regimen(28,33).This association was further described most recently by Rhee *et al* confirming the importance of the link between these two variables(52). We found that 51.8% of the patients on a TDF containing regimen at resistance test had the K65R mutation compared with 5.7% of patients on AZT and 7.7% of patients on D4T, confirming the expected association between TDF and K65R. In addition, 19.4% of the patients on TDF at resistance test also had the K70E mutation which has been shown to confer resistance to TDF. It was strongly associated with TDF exposure in our cohort. Aside from TDF being the most important risk factor for the K65R mutation, FTC being the second NRTI in the regimen was also associated with increased risk of having the K65R mutation in univariate analysis, but was protective in multivariate analysis. The univariate finding is likely explained by FTC only being available in a fixed dose combination with TDF in SA, thus always being accompanied by TDF in a regimen. Being on NVP at resistance test was not a significant risk factor in univariate analysis but was significantly associated with K65R in multivariate analysis. This may be related to NVP providing a less potent ART regimen than EFV(48).

TAMs were most commonly found in patients on an AZT containing regimens at resistance test (61.5%) and occurred less commonly in those on TDF based regimens (35.6%). The presence of the K65R mutation may have played a role in the decreased frequency of TAMs in those on TDF based regimens as these mutations are considered to be mutually exclusive in their occurrence(16,28). In univariate analysis duration on ART prior to resistance testing and duration documented to be failing ART were found to be significant factors associated with the presence of TAMs. Similar to our findings, Orrell *et al* reported that patients in their cohort tested for resistance beyond 6 months had a greater accumulation of TAMs, denoting the important association between duration on failing regimens and the prevalence of TAMs, with similar findings in other studies (17,29,40,49,50,55,56). Prior exposure to DDI, D4T and 3TC were also found to be important predisposing factors for TAMs. Ever being exposed to NVP was associated with presence of at least one NNRTI mutation. This is potentially important when considering the choice of NNRTI in first line (51).

Our study was conducted within the Southern African private sector and using data collected between 1998 and 2014. In 2010 the National Department of Health began to phase out the use of D4T due to its side effect profile and replaced it with TDF; this shift occurred slightly earlier in the private sector. As such over half the cohort time predated the introduction of TDF in first line. This had a role to play on the proportion of patients who developed some of the resistance mutations in question such as the K65R mutation. In our cohort, 51.8% of

patients failing tenofovir (TDF) regimens showed K65R (similar to the study by Sunpath *et al* (69.7%)) but K65R was observed in less than 10% of patients failing stavudine or zidovudine. We also found that the presence of TDF and NVP in a regimen was associated with the presence of the K65R mutation and this is consistent with various studies previously done(32,53). This has contributed to the preferred use of TDF/FTC/EFV as first line therapy as this combination has been shown to result in fewer cases of treatment failure (32,53).

Resistance testing was done in patients that were failing ART, which was defined as a viral load of > 1000 copies/ml on two consecutive occasions 24 weeks after ART commencement (15). In our cohort the viral load was unsuppressed for a median of 18 months. This means there was a prolonged period of exposure to a failing regimen allowing for the selection of TAMs and contributing to the high proportion of patients who had TAMs and who had resistance mutations in general on resistance testing. The long duration that patients were failing ART before resistance testing represents a bias in the selection of patients for resistance testing within the AfA programme. Particularly during the earlier years of resistance testing because of the cost these tests were generally restricted to patients with prolonged failure as it was anticipated they would have complex NRTI resistance patterns and thus the test would help in decisions regarding drugs to be included in second-line regimens. The findings are therefore not representative of all patients failing first-line ART in the private sector. A further limitation of the study was that data for certain variables was missing for a proportion of patients, especially baseline variables those who started ART before registered with AfA (see Table 1 footnote).

It is worth noting that the association noted between the V106M/A mutation and TDF is likely confounded due to the frequent co-administration of EFV with TDF, as they are co-formulated in fixed dose combinations tablets.

Conclusions

The key findings of this study are characterisation of the mutational patterns at first-line failure in the private sector in South Africa. There was a strong association between TDF and K65R; this mutation was seen in over 50% of the patients on TDF at first-line failure. K65R confers resistance to most NRTI's with the exception of AZT where it conversely increases susceptibility(6,28). This limits the NRTI choices available for second-line therapy, although recent clinical trials have questioned whether susceptible NRTI drugs are necessary for the virological efficacy of a lopinavir/ritonavir-based second-line regimen (19,20). There were few patients noted to have the Q151M mutation which may be associated with K65R (n=2) and when together result in pan-nucleoside resistance (6,28). Patients on TDF at resistance test were found to have the Y181C/I mutation more frequently than in AZT and D4T, (26.6% vs. 11.4% vs. 19.23% respectively) which has implications for susceptibility to the second generation NNRTI etravirine(46). As noted above these patients were virologically unsuppressed for a median of 18 months at time of resistance testing and duration unsuppressed was a risk factor for the development of TAMs. TAMs confer resistance to D4T and AZT and if patients are found to have more than 3 TAMs it causes resistance to most NRTI's available for second-line therapy.

We have shown that prolonged exposure to a failing first-line regimen is associated with very high proportion of patients having complex resistance mutation patterns. The roll-out of TDF appears to have substantially increased the proportion of patients with K65R at first-line failure, affecting the choices for future NRTIs in subsequent lines of therapy. Interestingly

TDF was associated with NNRTI mutations that have implications for etravirine in third-line therapy. Our findings paralleled the findings of studies done in the SA public sector regarding K65R. The use of resistance testing to identify mutational patterns at failure may be of benefit in facilitating subsequent regimen choices, but this has to be balanced against the cost in resource limited settings.

References

1. HIV/AIDS W. WHO | HIV/AIDS. World Health Organization; Available from: http://www.who.int/mediacentre/factsheets/fs360/en/
2. UNAIDS.org. Available from: http://www.unaids.org/
3. aidsinfo.unaids.org. Available from: http://aidsinfo.unaids.org/
4. Longo, Fauci, Kasper, Hauser, Jameson, Loscalzo. Harrison's Principles of Internal Medicine. 18th ed. McGraw-Hill;
5. Kumar, Abbas, Fausto, Aster. Robbins and Cotran Pathologic Basis of Disease. 8th ed. Saunders; 2009.
6. Meintjes G. Antiretroviral resistance. Continuing Medical Education 2008 p. 230.
7. Sethi, Ajay K.Celentano, David D.Gange, Stephen J.Moore, Richard D.1, 2Gallant, Joel E.1 2. Association between Adherence to Antiretroviral Therapy and Human Immunodeficiency Virus Drug Resistance.
8. Parienti J-J, Das-Douglas M, Massari V, Guzman D, Deeks SG, Verdon R, et al. Not all missed doses are the same: sustained NNRTI treatment interruptions predict HIV rebound at low-to-moderate adherence levels. PLoS One. Public Library of Science; 2008 Jul 30;3(7):e2783.
9. Mills EJ, Nachega JB, Bangsberg DR, Singh S, Rachlis B, Wu P, et al. Adherence to HAART: a systematic review of developed and developing nation patient-reported barriers and facilitators. PLoS Med. 2006 Nov;3(11):e438.
10. Gardner EM, Burman WJ, Maravi ME, Davidson AJ. Selective drug taking during combination antiretroviral therapy in an unselected clinic population. J Acquir Immune Defic Syndr. 2005 Nov 1;40(3):294–300.
11. Bangsberg DR, Kroetz DL, Deeks SG. Adherence-resistance relationships to combination HIV antiretroviral therapy. Curr HIV/AIDS Rep. 2007 Apr 26;4(2):65–72.
12. Hosseinipour MC, Gupta RK, Van Zyl G, Eron JJ, Nachega JB. Emergence of HIV drug resistance during first- and second-line antiretroviral therapy in resource-limited settings. J Infect Dis. 2013 Jun 15;207 Suppl:S49-56.
13. Ammassari A. Self-reported symptoms and medication side effects influence adherence to highly active antiretroviral therapy in persons with HIV infection. J Acquir Immune Defic Syndr. 2001;28(5).
14. Gupta S, Vingerhoets J, Fransen S, Tambuyzer L, Azijn H, Frantzell A, et al. Connection domain mutations in HIV-1 reverse transcriptase do not impact etravirine susceptibility and virologic responses to etravirine-containing regimens. Antimicrob Agents Chemother. 2011 Jun;55(6):2872–9.
15. Wallis CL, Papathanasopolous MA, Fox M, Conradie F, Ive P, Orrell C, et al. Low rates of nucleoside reverse transcriptase inhibitor resistance in a well-monitored cohort in South Africa on antiretroviral therapy. Antivir Ther. NIH Public Access; 2012 Jan;17(2):313–20.
16. Parikh UM, Bacheler L, Koontz D, Mellors JW. The K65R mutation in human immunodeficiency virus type 1 reverse transcriptase exhibits bidirectional phenotypic antagonism with thymidine analog mutations. J Virol. 2006 May;80(10):4971–7.
17. Hosseinipour MC, van Oosterhout JJG, Weigel R, Phiri S, Kamwendo D, Parkin N, et al. The public health approach to identify antiretroviral therapy failure: high-level nucleoside reverse transcriptase inhibitor resistance among Malawians failing first-line antiretroviral therapy. AIDS. NIH Public Access; 2009 Jun 1;23(9):1127–34.
18. Virological response to a triple nucleoside/nucleotide analogue regimen over 48 weeks in HIV-1-infected adults in Africa. AIDS, 2006 Jun 26;20(10):1391–9.
19. Paton NI, Kityo C, Hoppe A, Reid A, Kambugu A, Lugemwa A, et al. Assessment of second-line antiretroviral regimens for HIV therapy in Africa. N Engl J Med. 2014 Jul 17;371(3):234–47.

20. La Rosa AM, Harrison LJ, Taiwo B, Wallis CL, Zheng L, Kim P, et al. Raltegravir in second-line antiretroviral therapy in resource-limited settings (SELECT): a randomised, phase 3, non-inferiority study. Lancet HIV. 2016 Jun;3(6):e247-58.
21. Lessells RJ, Stott KE, Manasa J, Naidu KK, Skingsley A, Rossouw T, et al. Implementing antiretroviral resistance testing in a primary health care HIV treatment programme in rural KwaZulu-Natal, South Africa: early experiences, achievements and challenges. BMC Health Serv Res. 2014 Jan;14:116.
22. Corzillius M, Mühlberger N, Sroczynski G, Jaeger H, Wasem J, Siebert U. Cost effectiveness analysis of routine use of genotypic antiretroviral resistance testing after failure of antiretroviral treatment for HIV. Antivir Ther. 2004 Feb;9(1):27–36.
23. Sendi P, Günthard HF, Simcock M, Ledergerber B, Schüpbach J, Battegay M. Cost-effectiveness of genotypic antiretroviral resistance testing in HIV-infected patients with treatment failure. PLoS One. 2007 Jan;2(1):e173.
24. Lorenzana SB, Hughes MD, Grinsztejn B, Collier AC, Luz PM, Freedberg KA, et al. Genotype assays and third-line ART in resource-limited settings: a simulation and cost-effectiveness analysis of a planned clinical trial. AIDS. 2012 Jun 1;26(9):1083–93.
25. Manasa J, Katzenstein D, Cassol S, Newell M-L, de Oliveira T. Primary drug resistance in South Africa: data from 10 years of surveys. AIDS Res Hum Retroviruses. 2012 Jun;28(6):558–65.
26. Steegen K, Carmona S, Bronze M, Papathanasopoulos MA, van Zyl G, Goedhals D, et al. Moderate Levels of Pre-Treatment HIV-1 Antiretroviral Drug Resistance Detected in the First South African National Survey. Blackard J, editor. PLoS One. 2016 Dec 1;11(12):e0166305.
27. Oette M, Kaiser R, Däumer M, Petch R, Fätkenheuer G, Carls H, et al. Primary HIV drug resistance and efficacy of first-line antiretroviral therapy guided by resistance testing. J Acquir Immune Defic Syndr. 2006 Apr 15;41(5):573–81.
28. Tang MW, Rhee S-Y, Bertagnolio S, Ford N, Holmes S, Sigaloff KC, et al. Nucleoside reverse transcriptase inhibitor resistance mutations associated with first-line stavudine-containing antiretroviral therapy: programmatic implications for countries phasing out stavudine. J Infect Dis. 2013 Jun 15;207 Suppl:S70-7.
29. Orrell C, Walensky RP, Losina E, Pitt J, Freedberg KA, Wood R. HIV type-1 clade C resistance genotypes in treatment-naive patients and after first virological failure in a large community antiretroviral therapy programme. Antivir Ther. 2009 Jan;14(4):523–31.
30. Skhosana L, Steegen K, Bronze M, Lukhwareni A, Letsoalo E, Papathanasopoulos MA, et al. High prevalence of the K65R mutation in HIV-1 subtype C infected patients failing tenofovir-based first-line regimens in South Africa. Maga G, editor. PLoS One. 2015 Feb 6 ;10(2):e0118145.
31. Hoffmann CJ, Ledwaba J, Li J-F, Johnston V, Hunt G, Fielding KL, et al. Resistance to tenofovir-based regimens during treatment failure of subtype C HIV-1 in South Africa. Antivir Ther. NIH Public Access; 2013;18(7):915–20.
32. Van Zyl GU, Liu TF, Claassen M, Engelbrecht S, de Oliveira T, Preiser W, et al. Trends in Genotypic HIV-1 Antiretroviral Resistance between 2006 and 2012 in South African Patients Receiving First- and Second-Line Antiretroviral Treatment Regimens. Landay A, editor. PLoS One. Public Library of Science; 2013 Jun 26;8(6):e67188.
33. Sunpath H, Wu B, Gordon M, Hampton J, Johnson B, Moosa M-YS, et al. High rate of K65R for antiretroviral therapy-naive patients with subtype C HIV infection failing a tenofovir-containing first-line regimen. AIDS. 2012 Aug 24;26(13):1679–84.
34. Doualla-Bell F, Avalos A, Brenner B, Gaolathe T, Mine M, Gaseitsiwe S, et al. High prevalence of the K65R mutation in human immunodeficiency virus type 1 subtype C isolates from infected patients in Botswana treated with didanosine-based regimens. Antimicrob Agents Chemother. 2006 Dec;50(12):4182–5.
35. Cunningham CK, Chaix M-L, Rekacewicz C, Britto P, Rouzioux C, Gelber RD, et al. Development of resistance mutations in women receiving standard antiretroviral therapy who received intrapartum nevirapine to prevent perinatal human immunodeficiency virus type 1 transmission: a substudy of pediatric AIDS clinical trials group protocol 3. J Infect Dis. 2002 Jul 15;186(2):181–8.

36. Lockman S, Hughes MD, McIntyre J, Zheng Y, Chipato T, Conradie F, et al. Antiretroviral therapies in women after single-dose nevirapine exposure. N Engl J Med. 2010 Oct 14;363(16):1499–509.
37. Boltz VF, Bao Y, Lockman S, Halvas EK, Kearney MF, McIntyre JA, et al. Low-Frequency Nevirapine (NVP)-Resistant HIV-1 Variants Are Not Associated With Failure of Antiretroviral Therapy in Women Without Prior Exposure to Single-Dose NVP. J Infect Dis. 2014 Jan 16;209(5):703–10.
38. Eshleman SH, Mracna M, Guay LA, Deseyve M, Cunningham S, Mirochnick M, et al. Selection and fading of resistance mutations in women and infants receiving nevirapine to prevent HIV-1 vertical transmission (HIVNET 012). AIDS. 2001 Oct 19;15(15):1951–7.
39. Brehm JH, Koontz DL, Wallis CL, Shutt KA, Sanne I, Wood R, et al. Frequent emergence of N348I in HIV-1 subtype C reverse transcriptase with failure of initial therapy reduces susceptibility to reverse-transcriptase inhibitors. Clin Infect Dis. 2012 Sep;55(5):737–45.
40. Marconi VC, Sunpath H, Lu Z, Gordon M, Koranteng-Apeagyei K, Hampton J, et al. Prevalence of HIV-1 drug resistance after failure of a first highly active antiretroviral therapy regimen in KwaZulu Natal, South Africa. Clin Infect Dis. 2008 May 15;46(10):1589–97.
41. Paterson DLSS. Adherence to Protease Inhibitor Therapy and Outcomes in Patients with HIV Infection.
42. Bangsberg DR FH, Charlebois E, Zolopa A, Holodniy M, Sheiner L, Bamberger J, et al. Adherence to protease inhibitors, HIV-1 viral load, and development of drug resistance in an indigent population. AIDS. 2000;14(4):357–66.
43. Arnsten JH, Demas PA, Farzadegan H, Grant RW, Gourevitch MN, Chang CJ, et al. Antiretroviral therapy adherence and viral suppression in HIV-infected drug users: comparison of self-report and electronic monitoring. Clin Infect Dis. 2001 Oct 15;33(8):1417–23.
44. Harrigan, P. Richard1, 2Hogg, Robert S.1, 2Dong, Winnie W. Y.Yip, BenitaWynhoven, BrianWoodward, JustinBrumme, Chanson J.Brumme, Zabrina L.1, 2Mo, TheresaAlexander, Chris S.Montaner, Julio S. G.1 2. Predictors of HIV Drug-Resistance Mutations in a Large Antiretroviral-Naive Cohort Initiating Triple Antiretroviral Therapy.
45. LET OUR ACTIONS COUNT. 2012;2017–22. Available from: http://sanac.org.za/wp-content/uploads/2017/01/Final-NSP-Document.pdf
46. Wallis CL, Mellors JW, Venter WDF, Sanne I, Stevens W. Varied patterns of HIV-1 drug resistance on failing first-line antiretroviral therapy in South Africa. J Acquir Immune Defic Syndr. 2010 Apr 1;53(4):480–4.
47. Meintjes G. Antiretroviral resistance. Emergence of resistance. 2005;23(5).
48. Nachega JB, Hislop M, Dowdy DW, Gallant JE, Chaisson RE, Regensberg L, et al. Efavirenz versus Nevirapine-based Initial Treatment of HIV Infection: Clinical and Virological Outcomes in Southern African Adults. AIDS Oct. 2008;18(2216):2117–25.
49. Hosseinipour M, Kumwenda J, Weigel R, Brown L, Mzinganjira D, Mhango B, et al. Second-line treatment in the Malawi antiretroviral programme: high early mortality, but good outcomes in survivors, despite extensive drug resistance at baseline. HIV Med. Blackwell Publishing Ltd; 2010 Mar;11(8):510–8.
50. Kuritzkes DR, Bassett RL, Hazelwood JD, Barrett H, Rhodes RA, Young RK, et al. Rate of thymidine analogue resistance mutation accumulation with zidovudine- or stavudine-based regimens. J Acquir Immune Defic Syndr. 2004 May 1;36(1):600–3.
51. Lockman S, Hughes MD, McIntyre J, Zheng Y, Chipato T, Conradie F, et al. Antiretroviral therapies in women after single-dose nevirapine exposure. N Engl J Med. NIH Public Access; 2010 Oct 14;363(16):1499–509.
52. Rhee S-Y, Varghese V, Holmes SP, Van Zyl GU, Steegen K, Boyd MA, et al. Mutational Correlates of Virological Failure in Individuals Receiving a WHO-Recommended Tenofovir-Containing First-Line Regimen: An International Collaboration. EBioMedicine 2017;18:225–35.
53. Tang MW, Kanki PJ, Shafer RW. A Review of the Virological Efficacy of the 4 World Health Organization–Recommended Tenofovir-Containing Regimens for Initial HIV

Therapy. Clin Infect Dis. International AIDS Society, Sydney; 2012 Mar 15 [cited 2017 Jun 5];54(6):862–75
55. Sigaloff KCE, Ramatsebe T, Viana R, de Wit TFR, Wallis CL, Stevens WS. Accumulation of HIV drug resistance mutations in patients failing first-line antiretroviral treatment in South Africa. AIDS Res Hum Retroviruses. 2012 Feb ;28(2):171–5.
56. van Zyl GU, van der Merwe L, Claassen M, Zeier M, Preiser W. Antiretroviral resistance patterns and factors associated with resistance in adult patients failing NNRTI-based regimens in the western cape, South Africa. J Med Virol. Wiley Subscription Services, Inc., A Wiley Company; 2011 Oct;83(10):1764–9.

Table 1: Characteristics of patients

Variable	Number (%) or median (interquartile range)
Age in years, median (IQR)	37 (28.00 – 43.00)
< 10 years, n (%)	32/278 (11.51)
10 – 17 years, n (%)	21/278 (7.55)
>=18 years, n (%)	225/278 (80.94)
Gender, n (% female)	172/278 (61.87)
Province*, n (%)	
Eastern Cape	10/256 (3.91)
Free State	1/256 (0.39)
Gauteng	73/256 (28.52)
KwaZulu/Natal	110/256 (42.97)
Limpopo	25/256 (9.77)
Mpumalanga	23/256 (8.98)
North-West	13/256 (5.08)
Northern Cape	0/256 (0%)
Western Cape	1/256 (0.39)
WHO stage*, n (%)	
Stage 1	26/131 (19.85)
Stage 2	20/131 (15.27)
Stage 3	55/131 (41.98)
Stage 4	30/131 (22.90)
Duration of ART in months, median (IQR)	29 (15 - 53)
Monotherapy*, n (% yes)	9/226 (3.98)
Dual therapy*, n (% yes)	27/226 (11.95)
Drug at RT, n (% yes)	
AZT	88/278 (31.65)
D4T	26/278 (9.35)
TDF	139/278 (50.00)
ABC	23/278 (8.27)
DDI	2/278 (0.72)

3TC	123/278 (44.24)
FTC	138/278 (49.64)
DDI	17/278 (6.12)
EFV	227/278 (81.65)
NVP	51/278 (18.35)
Drug, ever on*, n (% yes)	
AZT	104/226 (46.02)
D4T	54/226 (23.89)
TDF	124/226 (54.87)
ABC	20/226 (8.85)
DDI	23/226 (10.18)
3TC	143/226 (63.27)
FTC	124/226 (54.87)
EFV	196/226 (86.73)
NVP	65/226 (28.76)
HIV viral load in copies/ml before ART, median (IQR)	235,000 (82,924 – 562,000)
CD4 count in cells/mm^3 before ART, median (IQR)	78 (27 - 178)
HIV viral load before RT, median (IQR)	85,688 (22,683 – 244,506)
CD4 count before RT, median (IQR)	115 (33.50 - 293.50)
Duration viral load > 400 copies/ml prior to resistance test,	18 (11 - 34)
Subtype	
C, n (%)	274/278 (98.56)
B, n (%)	1/278 (0.36)
C/B, n (%)	1/278 (0.36)
Other, n (%)	2/278 (72)

* n varies due to missing values

Footnote: 52 patients who were not registered with AfA when they initiated ART baseline data at the start of ART and data related to their early ART history was consistently missing – these patients transferred into AfA while already on ART. These 52 patients were not included in analysis of CD4 count and viral load before ART, WHO stage, previous mono/dual therapy, drugs ever on, duration on ART and duration failing ART (unless data could be accessed from narrative notes at time of entry into AfA which was available for a small number of these patients).

Table 2: Mutations present on resistance test

Mutations	**(n = 278)**
Any RT mutation	
n (% yes)	265 (95.32)
Median (IQR)	4 (3 – 5)
TAMS	
n (% yes)	123 (44.24)
Median (IQR)	2 (1 – 3)
NRTI	
n (% yes)	246 (88.49)
Median (IQR)	2 (1 – 3)
NNRTIs	
n (% yes)	253 (91.01)
Median (IQR)	2 (1 – 2)
K65R	
n (% yes)	83 (29.86)
Median (IQR)	0 (0 – 1)

Table 3: Mutations associated with tenofovir (TDF) exposure

Mutations	Exposed to TDF (n=150; %)	Unexposed to TDF (n=128; %)	p-value
NRTIs			
T69INS	-	-	-
Q151M	2 (1.33)	3 (2.34)	0.664
M41L	8 (5.33)	36 (28.12)	<0.001
D67N	28 (18.67)	38 (29.69)	0.031
K70R	11 (7.33)	33 (25.78)	<0.001
L210W	0 (0.00)	5 (3.91)	0.020
T215Y/F	10 (6.67)	47 (36.72)	<0.001
K219Q/E	24 (16.00)	31 (24.22)	0.086
K65R	72 (48.00)	11 (8.59)	<0.001
K70E	27 (18.00)	1 (0.78)	<0.001
L74V	6 (4.00)	15 (11.72)	0.015
Y115F	18 (12.00)	10 (7.81)	0.248
M184V/I	128 (85.33)	96 (75.00)	0.030
NRTIs, n (% yes)	134 (89.33)	112 (87.50)	0.633
Total number NRTI mutations, median (IQR)	2(1 - 3)	2.5 (1 - 4)	0.152
TAMS			
TAMs, n (% yes)	49 (32.67)	74 (57.81)	<0.001
Total TAMS, median (IQR)	2 (1 – 3)	3 (1 - 4)	0.110
NNRTIs			
L100I	16 (10.67)	4 (3.12)	0.019
K101P	3 (2.20)	3 (2.34)	1.000
K103N/S	61 (40.67)	56 (43.75)	0.604
V106A/M	65 (43.33)	40 (31.25)	0.038
V108I	15 (10.00)	11 (8.59)	0.688
Y181C/I	37 (24.67)	20 (15.62)	0.063
Y188C/L/H	18 (12.00)	20 (15.62)	0.381
G190S/A	48 (32.00)	34 (26.56)	0.322
P225H	13 (8.67)	15 (11.72)	0.399
M230L	6 (4.00)	4 (3.12)	0.757
NNRTIS, N (%)	137 (91.33)	116 (90.62)	0.837
Total number NNRTI mutations, median (IQR)	2 (1 – 2)	2 (2 – 2)	0.007

* n varies due to missing values

Table 4: NRTI drug at resistance test and mutation frequency

Mutations	TDF (n=139)	AZT (n=88)	D4T (n=26)	Exposed to Other (n=25)	p-value
NRTIs, n[¥] (%)					
T69INS	-	-	-	-	
Q151M	2 (1.44)	0 (0.00)	3 (11.54)	0 (0.00)	0.009
M41L	6 (4.32)	29 (33.95)	7 (26.92)	2 (8.00)	<0.001
D67N	25 (17.99)	32 (36.36)	6 (23.01)	3 (12.00)	0.008
K70R	9 (6.47)	27 (30.68)	5 (19.23)	3 (12.00)	<0.001
L210W	0 (0.00)	4 (4.55)	1 (3.85)	0 (0.00)	0.035
T215Y/F	7 (5.04)	40 (45.45)	7 (26.92)	3 (12.00)	<0.001
K219Q/E	21 (15.11)	25 (28.41)	5 (19.23)	4 (16.00)	0.108
K65R	72 (51.80)	5 (5.68)	2 (7.69)	4 (16.00)	<0.001
K70E	27 (19.42)	1 (1.14)	0 (0.00)	0 (0.00)	<0.001
L74V	2 (1.44)	1 (1.14)	1 (3.85)	17 (68.00)	<0.001
Y115F	15 (10.79)	1 (1.14)	1 (3.85)	11 (44.00)	<0.001
M184V/I	119 (85.61)	66 (75.00)	17 (65.38)	22 (88.00)	0.037
NRTIs, n (%yes)	125 (89.93)	75 (85.23)	23 (88.46)	23 (92.00)	0.728
TOTAL NRTIS, median (IQR)	2 (1 – 3)	3 (1 – 4)	2 (1 – 3)	3 (2 – 3)	0.128
TAMS, n (%yes)	44 (31.65)	57 (64.77)	16 (61.54)	6 (24.00)	<0.001
Total TAMS median (IQR)	2 (1 – 3)	3 (1 – 4)	2 (1 – 3)	3 (2 – 4)	0.067
NNRTIS, n (%)					
L100I	16 (11.51)	2 (2.27)	1 (3.85)	1 (4.00)	0.046
K101P	3 (2.16)	2 (2.27)	1 (3.85)	0 (0.00)	0.827
K103N/S	56 (4. 29)	42 (47.73)	11 (42.31)	8 (32.00)	0.498
V106A/M	62 (44.60)	24 (27.27)	11 (42.31)	8 (32.00)	0.058
V108I	13 (9.35)	8 (9.09)	3 (11.54)	2 (8.00)	0.972
Y181C/I	37 (26.62)	10 (11.36)	5 (19.23)	5 (20.00)	0.052
Y188C/L/H	14 (10.07)	12 (13.64)	7 (26.92)	5 (20.00)	0.100
G190S/A	45 (32.37)	25 (28.41)	6 (23.08)	6 (24.00)	0.686
P225H	12 (8.63)	13 (14.77)	2 (7.69)	1 (4.00)	0.394
M230L	6 (4.32)	2 (2.27)	1 (3.85)	1 (4.00)	0.768
NNRTIs, n (%yes)	127 (91.37)	81 (92.05)	24 (92.31)	21 (84.00)	0.625
Number of NNRTI mutations median (IQR)	2 (2 – 2)	2 (1 – 2)	2 (2 – 2)	2 (1 – 2)	0.010

[¥]n varies due to missing values

Footnote: This table summarises data according to the first NRTI drug in the ART regimen at resistance test. For the majority of patients, the second drug at resistance test was 3TC or FTC, and these were not included in this analysis.

Supplementary table 1: Unadjusted risk ratios for variables associated with TAMs

	Univariate	
Variable	**RR (95%CI)**	**P value**
Age group*		
10 -18 years	1.02 (0.63 – 1.64)	0.949
>18 years	0.73 (0.52 – 1.04)	0.078
Gender**	0.87 (0.66 – 1.13)	0.303
Duration ART (log)	2.32 (1.69 – 3.17)	<0.001
Duration failing (log) **Duration VL>400**	1.60 (1.18 – 2.16)	0.002
Drug 1 at RT† (ref: AZT)		
D4T	0.95 (0.68 – 1.34)	0.768
Other	0.37 (0.18 – 0.76)	0.006
TDF	0.49 (0.370 – 0.65)	<0.001
Drug 2 at RT†† (ref: 3TC)		
FTC	0.61 (0.46 – 0.82)	0.001
DDI	1.70 (1.33 – 2.16)	<0.001
Drug 3 at RT¶ (ref: EFV)		
NVP	1.19 (0.88 – 1.62)	0.262
Drug, ever on§		
AZT	1.89 (1.44 – 2.5)	<0.001
D4T	1.46 (1.13 – 1.90)	0.004
TDF	0.57 (0.43 – 0.74)	<0.001
ABC	0.36 (0.14 – 0.88)	0.025
DDI	2.05 (1.63 – 2.58)	<0.001
3TC	1.42 (1.05 – 1.93)	0.023
FTC	0.57 (0.44 – 0.75)	<0.001
EFV	0.79 (0.57 – 1.10)	0.162
NVP	1.14 (0.86 – 1.51)	0.349

* Reference group 4 -10 years
** Reference group male
† Reference group AZT
†† Reference group 3TC
¶ Reference group EFV
§ Reference group 'never on'

Supplementary table 2: Unadjusted and adjusted risk ratios for variables associated with K65R

	Univariate		Multivariable	
Variable	**RR (95%CI)**	**P value**	**RR (95%CI)**	**P value**
Age group*				
10-18 years	1.02 (0.19 - 5.57)	0.986	1.31 (0.25- 6.92)	0.752
>18 years	3.70 (1.24 - 1.02)	0.019	0.80 (0.18-3.54)	0.771
Gender**	1.43 (0.96 - 2.14)	0.081	0.98 (0.69-1.39)	0.901
Duration ART (log)	0.63 (0.98 - 0.99)	0.033	1.23 (0.82-1.85)	0.321
Duration failing (log) **Duration VL>400**	0.80 (0.54 – 1.18)	0.255	1.12 (0.79-1.57)	0.532
Drug 1 at RT † (ref: AZT)				
D4T	1.35 (0.28 - 6.58)	0.707	2.07 (0.34-12.55)	0.430
Other	2.82 (0.82 - 9.71)	0.101	4.95 (1.11-21.99)	0.036
TDF	9.12 (3.83 - 21.68)	<0.001	30.54 (8.15-114.5)	<0.001
Drug 2 at RT†† (ref: 3TC)				
FTC	5.75 (3.20 - 10.34)	<0.001	0.63 (0.42-0.94)	0.022
DDI	0.66 (0.09 - 4.78)	0.679	0.81 (0.10-6.26)	0.837
Drug 3 at RT¶ (ref: EFV)				
NVP	1.06 (0.68 - 1.67)	0.792	1.59 (1.17-2.17)	0.003
Drug, ever on§				
AZT	0.51 (0.34 - 0.76)	0.001		
D4T	0.58 (0.35 – 0.99)	0.045		
TDF	5.59 (3.10 – 10.07)	<0.001		
ABC	0.54 (0.22 – 1.34)	0.181		
DDI	0.45 (0.18 – 1.14)	0.093		
3TC	0.50 (0.35 – 0.71)	<0.001		
FTC	5.12 (2.19 – 9.01)	<0.001		
EFV	1.34 (0.73 – 2.45)	0.340		
NVP	1.21 (0.83 – 1.78)	0.323		

* Reference group 4 -10 years
** Reference group male
† Reference group AZT
†† Reference group 3TC
¶ Reference group EFV
§ Reference group 'never on'

Footnote: 'A priori' risk factors: Age, gender, duration ART, duration failing, drugs (ever, drug at resistance)

Supplementary table 3: Unadjusted risk ratios for variables associated with NNRTI mutations regression

	Univariate	
Variable	**RR (95%CI)**	**P value**
Age group*		
10-18 years	0.10 (0.84 – 1.19)	0.986
>18 years	1.01 (0.89 – 1.19)	0.930
Gender**	1.06 (0.98 – 1.15)	0.162
Duration ART (log)	1.06 (0.97 - 1.16)	0.161
Duration failing (log) Duration VL > 400 copies/ml	1.06 (0.97 - 1.16)	0.108
Drug1 at RT† (ref: AZT)		
D4T	1.00 (0.88 – 1.14)	0.965
Other	0.91 (0.76 – 1.09)	0.324
TD4	0.99 (0.92 – 1.08)	0.856
Drug2 at RT†† (ref: 3TC)		
FTC	1.00 (0.93 – 1.08)	0.944
DDI	0.97 (0.81 – 1.16)	0.735
Drug3 at RT¶ (ref: EFV)		
NVP	1.04 (0.96 – 1.13)	0.96
Drug, ever on§		
AZT	1.02 (0.95 – 1.10)	0.521
D4T	1.07 (0.99 - 1.14)	0.069
TDF	1.01 (0.94 – 1.09)	0.837
ABC	0.91 (0.76 – 1.09)	0.303
DDI	1.02 (0.92 – 1.14)	0.690
3TC	1.03 (0.95 – 1.12)	0.468
FTC	1.01 (0.93 – 1.08)	0.867
EFV	0.98 (0.89 – 1.09)	0.744
NVP	1.08 (1.01-1.15)	0.027

* Reference group 4 -10 years
** Reference group male
† Reference group AZT
†† Reference group 3TC
¶ Reference group EFV
§ Reference group 'never on'

ART abbreviations across tables:
AZT = zidovudine; D4T = stavudine; TDF = tenofovir; ABC = abacavir; DDI = didanosine; 3TC = lamivudine; FTC = emtricitabine; EFV = efavirenz; NVP = nevirapine.

RT = resistance test

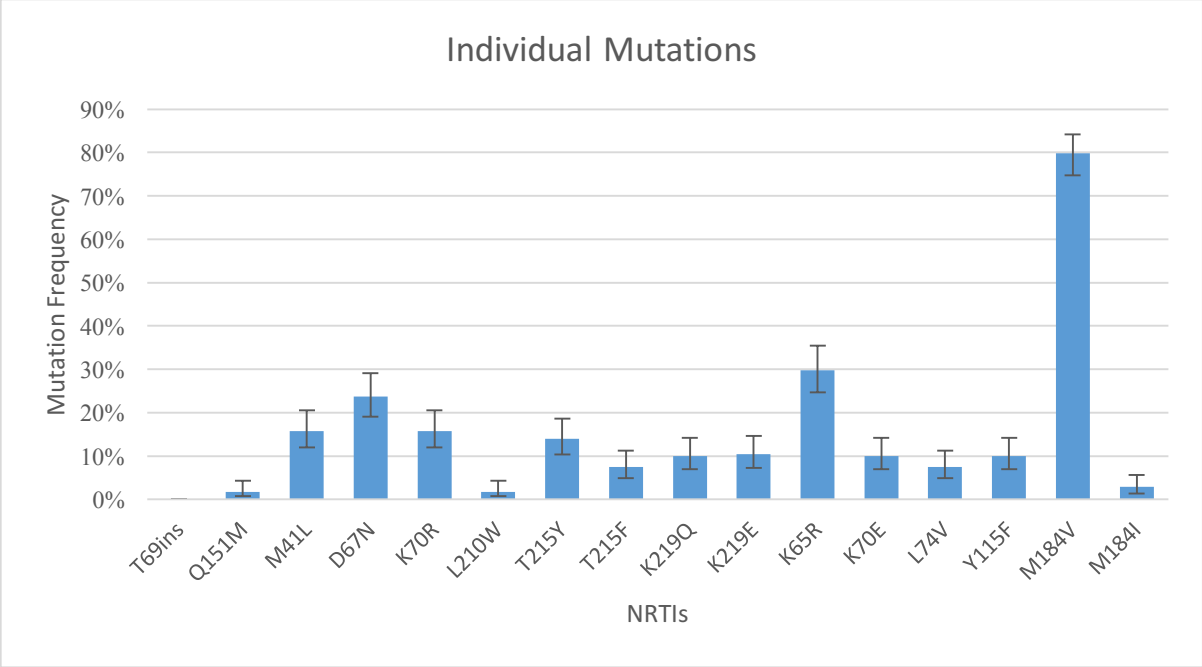


Figure 1: Frequency of individual nucleoside reverse transcriptase inhibitor (NRTI) mutations

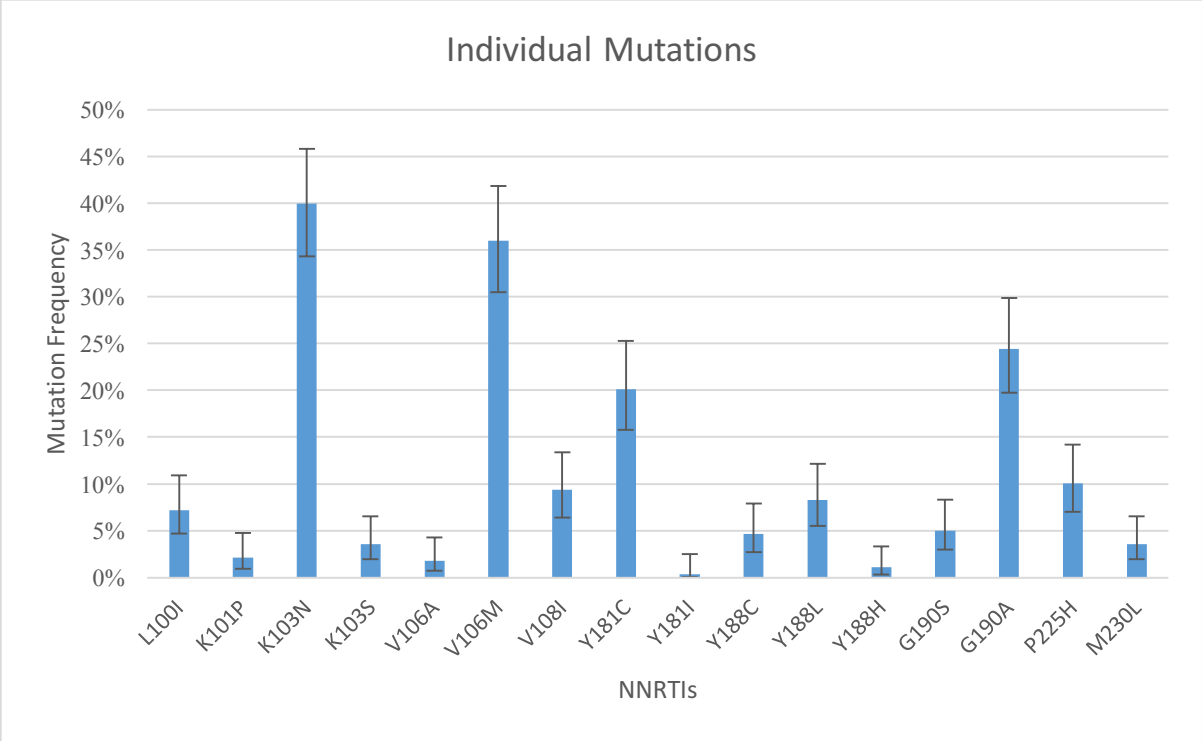


Figure 2: Frequency of individual non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations

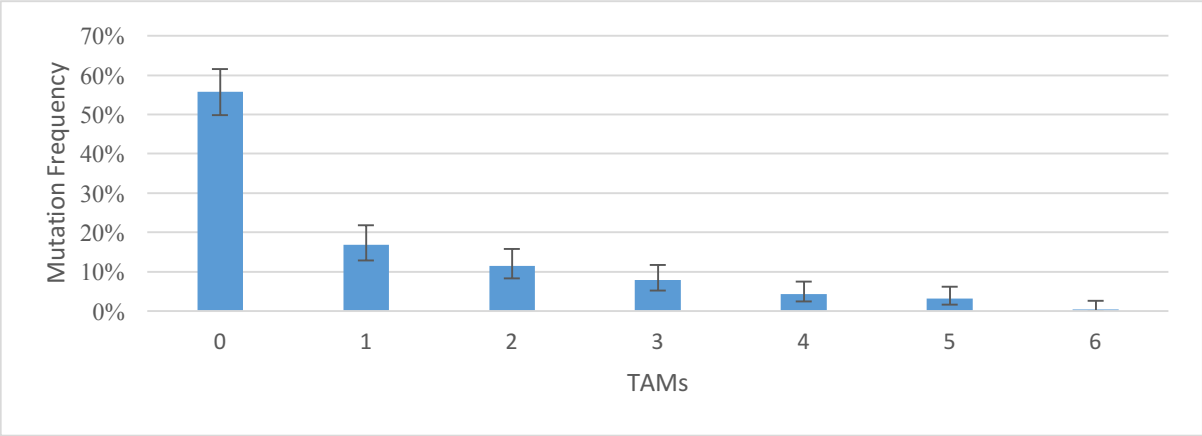


Figure 3: Frequency of thymidine analogue mutations (TAMs) by number present

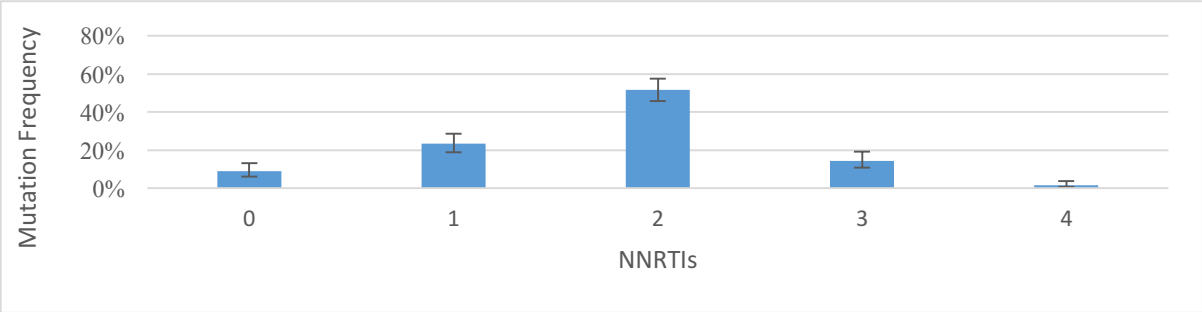
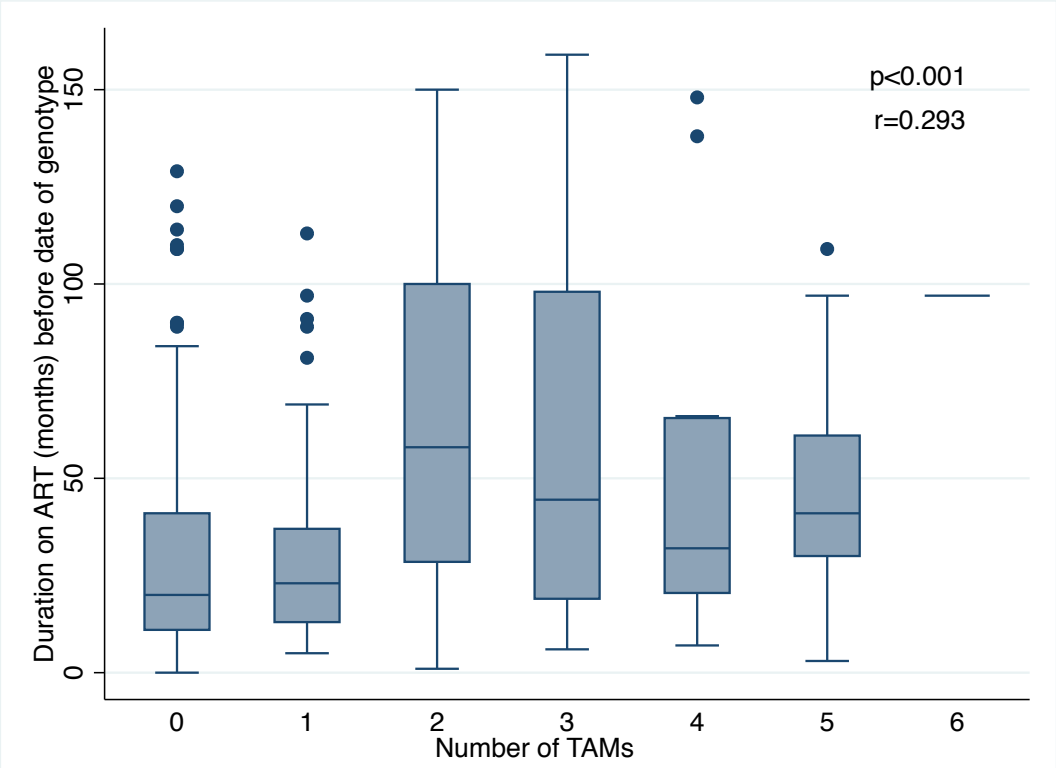
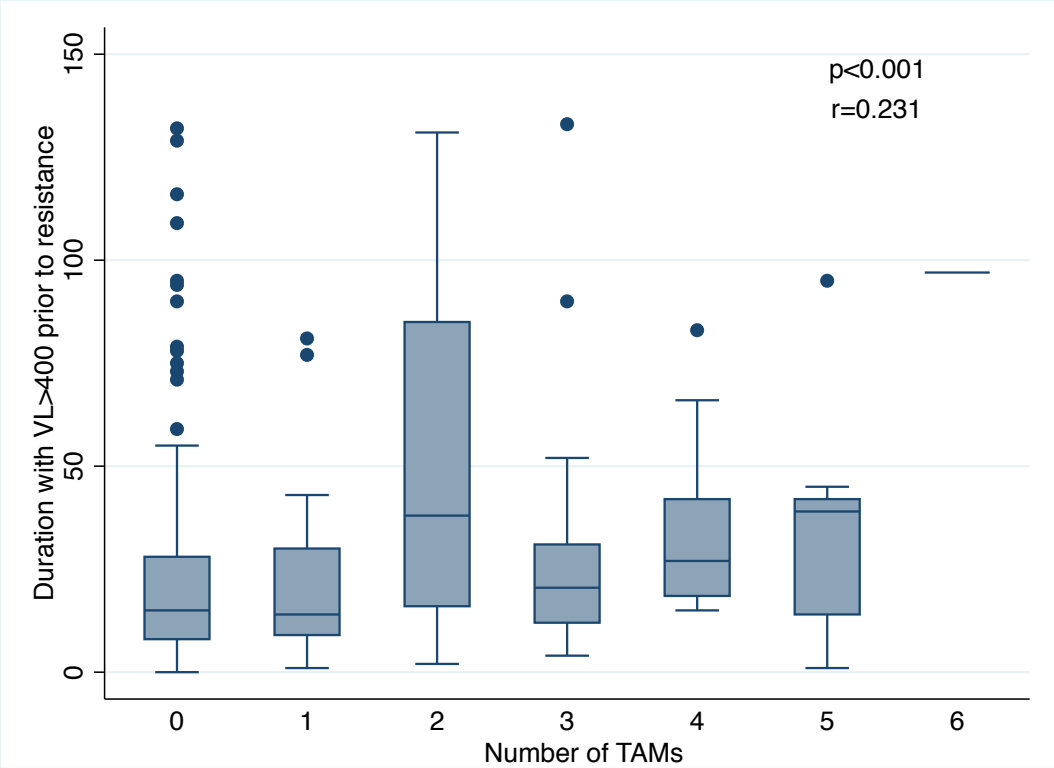


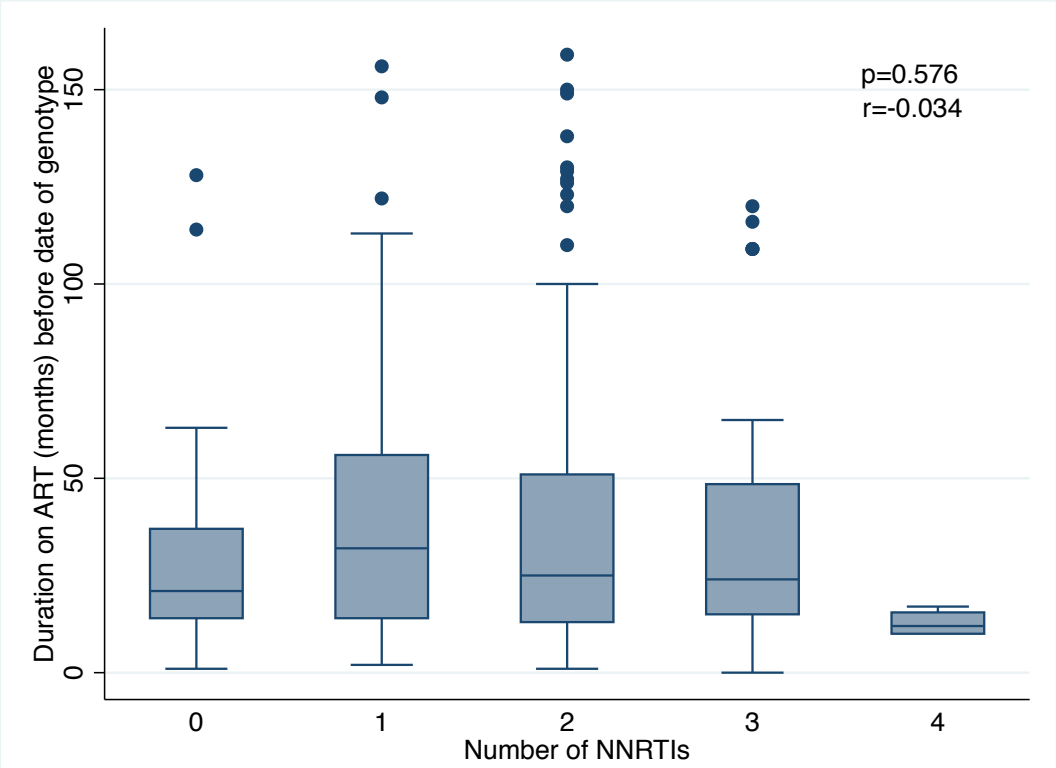
Figure 4: Frequency of non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations by number present



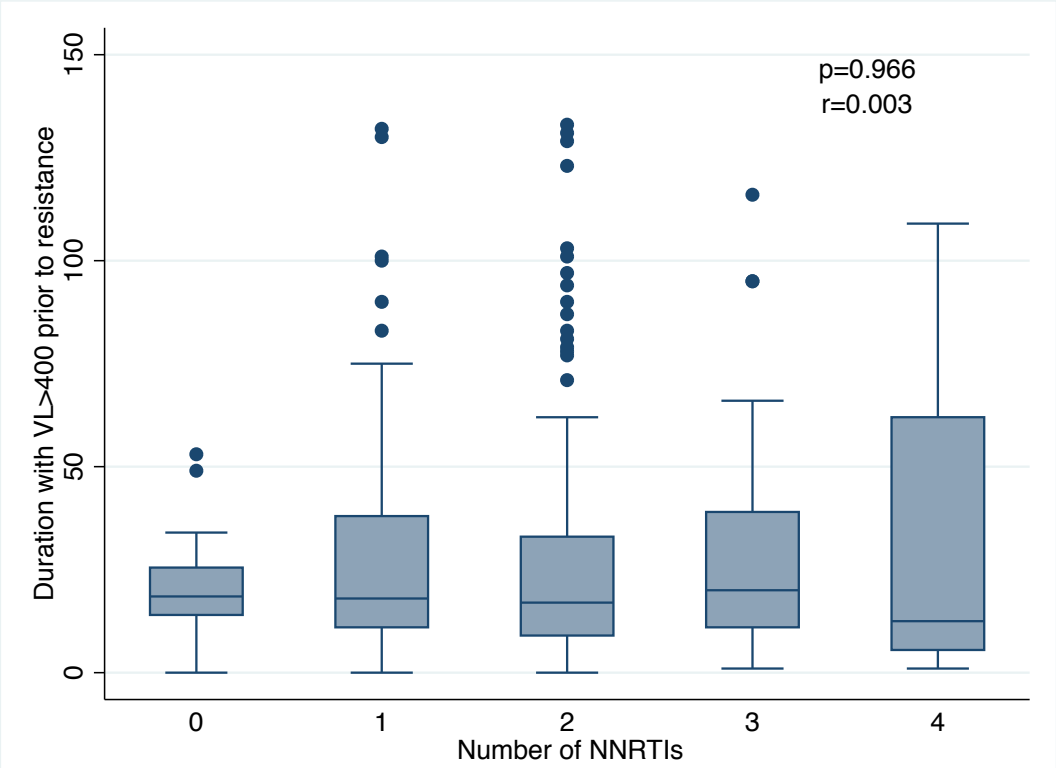
Supplementary figure 1: Duration on ART in relation to number of thymidine analogue mutations (TAMs) present on resistance test



Supplementary figure 2: Duration failing ART (viral load > 400 copies/ml) in relation to number of thymidine analogue mutations (TAMs) present on resistance test



Supplementary figure 3: Duration on ART in relation to number of non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations present on resistance test



Supplementary figure 4: Duration failing ART (viral load > 400 copies/ml) in relation to number of non-nucleoside reverse transcriptase inhibitor (NNRTI) mutations present on resistance test